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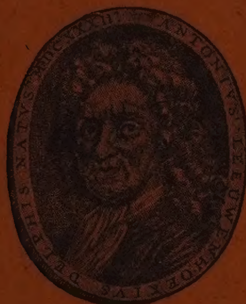

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ON THE ACTION OF SULFANILAMIDE

X. THE MECHANISM OF ACTION OF SULFANILAMIDE DERIVATIVES IN VITRO [1]

by

H. W. JULIUS and K. C. WINKLER

(Received May 27, 1943).

A. Introduction.

The mechanism of action of sulfanilamide in vitro differs widely from the action of germicides in general, which the following characteristic features render evident:

a) sulfanilamide is not directly bactericidal; most bacteria survive for 24 hours and longer even in excessive concentrations. A RIDEAL-WALKER coefficient cannot be determined.

b) the action of the drug occurs only in growing cultures, inoculated with a not over large number of bacteria and does not become manifest until some hours of normal growth have elapsed ("lag time of action" c.f. fig. 2 and fig. 3).

c) addition of blood or serum, so deleterious on the effect of other desinfectants seems not to decrease this sulfanilamide action, but even to enhance it under certain circumstances.

d) the action of sulfanilamide is counteracted (specifically?) by very small concentrations of p. aminobenzoic acid.

With regard to the mechanism of action of the compounds of sulfanilamide it soon became evident that all drugs of the sulfamide-group actually show the same features and it is quite evident that in vitro at least, the mechanism of action within this group is essentially the same.

Still it is beyond doubt that some of the compounds of sulfanilamide (sulfapyridine, sulfathiazol a.o.) are more potent drugs than sulfanilamide itself. It is one of the objects of this paper to contribute to our knowledge of this problem.

In no. III of this series one of us (2) showed, for sulfapyridine, that apart from the sulfanilamide-action, so far as in vivo conditions are concerned, another activity must be present in the molecule. Some evidence was produced, that this activity involved the defence of the host, though it was not possible to elucidate, in which way this might be realised.

A better quantitative analysis of the in vitro conditions showed, however, that also in vitro there does exist a distinct quantitative difference in the activity of sulfanilamide and its various compounds, thus providing an additional explanation for differences in vivo. Differences in toxicity,

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[1]) Ninth communication: K. C. WINKLER, Antonie van Leeuwenhoek **9**, 115, 1943.

solubility and many of the like of course also have to be taken in account for explaining the greater effectiveness of some of the heterocyclic derivatives in vivo.

In this paper we will limit ourselves strictly to the in vitro action of sulfanilamide and its derivatives. The mechanism of action being the same, a higher activity in vitro of the derivatives as compared with sulfanilamide can only mean: the same activity in lower concentrations. Indeed in growth experiments the difference between sulfapyridine and sulfanilamide appears to be no other than a difference in concentration (Exp. I).

The question thus arises: why smaller amounts of sulfapyridine are equally active as sulfanilamide, the mechanism of action being the same.

B. Activity and adsorption.

p.Aminobenzoic acid inhibits the action of sulfanilamide to such an

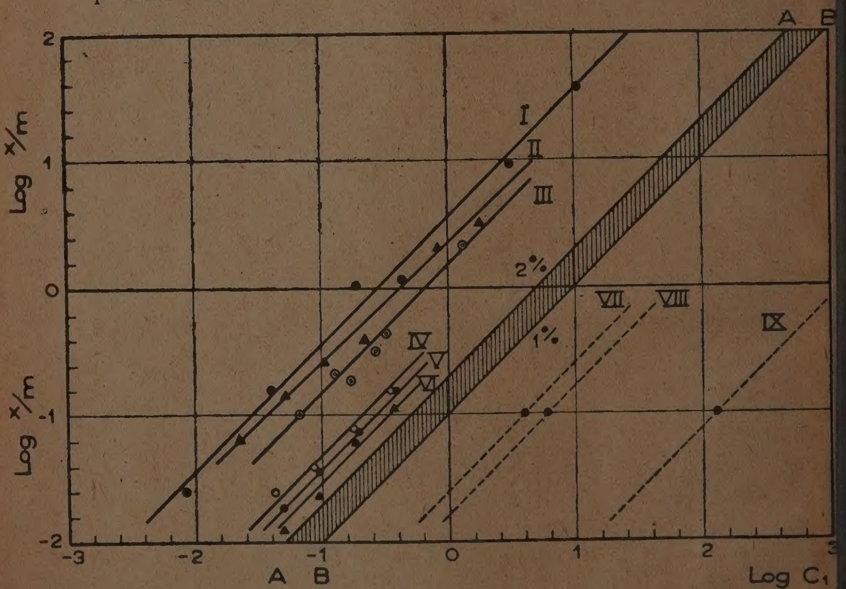


Fig. 1. Adsorption of various sulfonamides by *B. coli*.

log x/m = adsorbed quantity in m.mol. per litre of bacteria.
log C_1 = equilibrium concentration in m.mol. per litre.

I. p. aminobenzoic acid — II. methylsulfathiazol
III. sulfathiazol — IV. 2-sulfanilamido-4-methylpyrimidine
V. sulfapyridine — VI. 5-sulfanilamido-4-methylpyrimidine
A—A. limit of measurableness for an experimental error of 2 %
B—B. limit of measurableness for an experimental error of 1 %
VII. supposed curve for both the sulfanilamido-pyrazoles
VIII. supposed curve for sulfanilamide
IX. supposed curve for sulfanilic acid

extent that the counteracting concentration in the case of *B. coli* is 1200 times smaller than the sulfanilamide concentration which was active (3). As, in the end one molecule of p.aminobenzoic acid can compete with merely one molecule of sulfanilamide, the difference in active concentrations has to be explained; f.i. by an adsorption process. That means, that the adsorbed quantity of both substances should be equal at very different concentrations. In fact we found (3) that p.aminobenzoic acid is actively adsorbed by *B. coli*, whilst sulfanilamide (within the experimental error) is not.

Considering this fact it might be supposed that the greater activity of sulfapyridine (and other derivatives) also could be due to a better adsorption in the bacteria.

To produce corroborating evidence we studied in how far the activity of thirteen sulfanilamide compounds for *B. coli* was related to their adsorption to this organism.

The activity was studied by adding various concentrations of each drug to a synthetic medium, inoculated with *B. coli* and determining the s m a l l-e s t e f f e c t i v e c o n c e n t r a t i o n. The viable count was used as a measure in preference to the turbidity which is often used in this kind of experiments, but which is not sensitive enough for smaller numbers of bacteria and does not differentiate living bacteria from dead. Moreover the form of the growth curves (f.i. fig. 3) shows that the essentials of the process will be missed if turbidity is used as a measure.

The a d s o r p t i o n of sulfanilamide or its derivatives was determined by adding various concentrations of the used drug to concentrated suspensions of *B. coli* and after centrifuging, estimating the concentration of the drug in the supernate, by diazotization and coupling to oxychinoline.

Experiment I for instance bears on the determination of the activity of sulfapyridine, whilst in Experiment II some instances of the determination of adsorption are given. The results are collected in table I. In table I, columm a, the minimal active concentrations of the studied drugs are given, whereas the relative activity with regard to sulfanilamide can be found in column b. It is evident for instance from these figures that sulfapyridine is 30 times, sulfathiazol 75 times more active than sulfanilamide and so on.

The results of the adsorption experiments are presented in fig. 1. The log of the adsorbed quantity per unit of bacteria (log x/m) is plotted against the log of the equilibrium-concentration in m.moles per l. All curves appear to be parallel to each other with an inclination of 45°. p.Aminobenzoic acid appears to be adsorbed better than any of the sulfanilamides under discussion. Ordering the latter in the order of sequence of decreasing adsorption the following series is obtained:

Methylsulfathiazol > sulfathiazol > 2-sulfanilamido-4-methylpyrimidine > sulfapyridine > 5-sulfanilamido-4-methylpyrimidine

The sulfapyrazoles, sulfanilamide itself and sulfanilic acid were not adsorbed within the experimental error (see below).

From the above experiments it appears that the order of sequence of increasing adsorption is the same as the order of sequence for increasing activity. Indeed a quantitative correlation between activity and adsorbability is evident from table I column c, where the adsorbed quantity,

Table I.

All concentrations are given in m. mol. per litre.

Column a: minimal concentration of the drug which is active i. e. reduces the number of viable bacteria below 10 per standard droplet i.e. 350 per ml in 24 hours (Exp. I).

Column b: activity coefficient with regard to sulfanilamide.

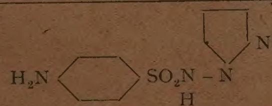
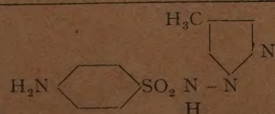
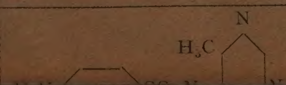
Column c: adsorbed quantity x/m in m. mol. per litre of bacteria (Exp. II) for C_1 = minimal active concentration.

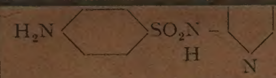
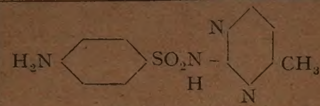
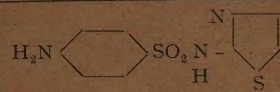
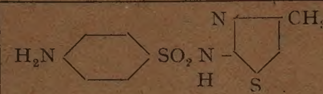
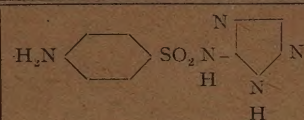
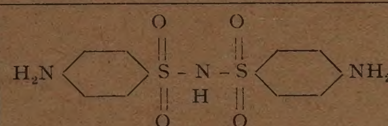
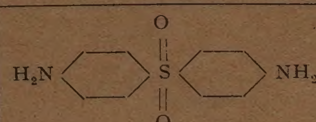
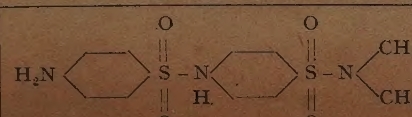
Column d: smallest concentration of p. aminobenzoic acid which is completely inhibiting the effect of the minimal active concentration of the drug (Exp. III).

Column e: a/d = ratio between active concentrations of p. aminobenzoic acid and sulfanilamide or related drugs.

Column f: b × e = 6/a × a/d = 6/d = constant.

□ = no adsorption within the experimental error.

Name	*Formula*	*a*	*b*	*c*	*d*	*e*	*f*
Sulfanilic acid		116	0.05	□	0.005	23000	~ 1200
Sulfanilamide		6	1	□	0.005	1200	1200
2-Sulfanilamido-pyrazol		4	1.5	□	0.005	800	1200
2-Sulfanilamido-4-methylpyrazol		4	1.5	□	0.005	800	1200
5-Sulfanilamido-4-		0.35	17	0.11	0.005	70	1200

Sulfapyridine		0.20	30	0.08	0.005	40	1200
2-Sulfanilamido-4-methylpyrimidine		0.15	40	0.07	0.005	30	1200
Sulfathiazol		0.08	75	0.12	0.005	16	1200
Sulfanilamido-methylthiazol (Methylsulfathiazol)		0.07	85	0.15	0.005	14	1200
Sulfatriazol		inactive > 4		□			
Di-sulfanilamide		inactive > 4		□			
Diamino-diphenyl-sulfon		0.8	7.5	4	0.005	160	1200
„Uliron"		inactive > 0.028 (saturated)		strongly adsorbed			

which corresponds with the concentration, which is the minimum active in the growth experiments, is given. It is evident that the adsorbed quantities at active concentrations are equal (about 0.1 m.mol.).

This probably holds also for sulfanilamide itself, but escapes experimental corroboration, as the adsorbed amount (about 0.1 m.mol.) with a 10 % suspension of bacteria, only reduces the outer concentration from 6 m.mol. to 5.99 m.mol. This difference is within the experimental error. Supposing an experimental error of about 2 %, any adsorbed quantity represented by a point to the right of the straight line A-A in fig. 1 falls within this error. The adsorption by sulfanilamide f.i. might be represented by any (straight) line to the right of A-A. So the dotted curves drawn for the sulfapyrazoles, sulfanilamide and sulfanilic acid in fig. 1 are purely hypothetical of course. They were drawn, however, on the surmisal that for the latter four drugs the adsorbed quantity at the active concentration would also be about 0.1 m.mol.

Sulfatriazol and disulfanilamide, which are inactive in our growth experiments are, as was to be expected, not adsorbed.

From this quantitative correlation between activity and adsorbability one might conclude that the better action of the studied sulfanilamide compounds in vitro is due to a better adsorption.

Only two out of the thirteen substances studied did not show the described correlation. Diamino-diphenylsulfon is as strongly adsorbed as p. aminobenzoic acid and is only 7.5 times as active as is sulfanilamide, whilst Uliron which is adsorbed about as strongly as sulfathiazol, at its saturated concentration is not active at all in our experiments with *B. coli* (though it is for streptococci in vitro and in vivo).

The structural formulae of these substances might explain this: an a-specific adsorption at inactive centres or some kind of sterical hindrance might affect the effectivity of adsorbed molecules in such a case.

The fact that the action of diamino-diphenylsulfon is inhibited by the same concentration of p. aminobenzoic acid, which inhibits sulfanilamide action, corroborates this view (see section C).

C. The inhibition of the action of various sulfanilamides by p.aminobenzoic acid.

As sulfapyridine is thirty times more active than sulfanilamide, what is to be expected for the counteracting concentration of p.aminobenzoic acid? Will the concentration in which this inhibitor just counteracts the drug also decrease, or will it be equal for sulfanilamide and sulfapyridine?

As the better action of sulfapyridine is due to better adsorption, this means, that in one thirtieth of the concentration of the sulfanilamide, the sulfapyridine is able to occupy an equal number of centres of adsorption as does sulfanilamide.

It is reasonable to suppose, that for complete inhibition of the sulfanilamide or sulfapyridine effect an equal number of p.aminobenzoic acid molecules will have to be adsorbed at these centres. Such an adsorption will occur at a given p.aminobenzoic acid concentration and will be (practically) independent of the kind of sulfanilamide compound to be displaced.

So it may be expected that the counteracting concentration of p.aminobenzoic acid will be equal for all sulfanilamide compounds.

We studied this counteraction of p.aminobenzoic acid by adding various concentrations of this substance to media, inoculated with *B. coli*, which contained the minimal active concentration of the studied sulfanilamide compounds and by determining the minimal p.aminobenzoic acid concentration that re-established normal growth. Here again the viable count was used as a measure. In Exp. III some instances of these experiments are given (Fig. 4 and 5).

In table I, column d, the minimal inhibiting p.aminobenzoic acid concentrations are given. The concentration in which p. aminobenzoic acid counteracts appears to be equal for all the drugs studied.

In column e the ratio between the minimal active concentration of the sulfanilamide compound (column a) and the corresponding p. aminobenzoic acid concentration (column d) is calculated. For sulfanilamide this ratio is 1200, for sulfapyridine, which is 30 times as active the ratio is 40. The product of the activity quotient (column b) and the discussed ratio has to be constant of course ($30 \times 40 = 1200$). This shows that the ratio e has no vital meaning, but is dependent on the activity of the sulfanilamide compound. The really important fact is the constancy of the concentration in which p. aminobenzoic acid counteracts all the compounds studied.

From this fact it follows, that the number of adsorbed p.aminobenzoic acid molecules, which are necessary for the displacement of the sulfonamide, is the same for each compound. So it is evident, that the number of molecules displaced is also equal for each of the various compounds and this means, that at the different minimal active concentrations, the adsorbed quantities are equal.

From the experimental fact that the minimal concentration, in which p.aminobenzoic acid counteracts, is the same for all sulfonamides, we can independently again arrive at our former conclusion, that the different activity of these compounds is due to differences in adsorbability.

It would seem probable that the adsorbed quantity of p. aminobenzoic acid at the inhibiting concentration of 0.05 m.mol. should be also 0.1 m.mol. per litre of bacteria. Unfortunately this quantity can only be found by extrapolation. According to fig. 1 the adsorbed quantity of p.aminobenzoic acid per litre of bacteria should be 0.02 m. mol. Though this difference might be explained by the extrapolation and the experimental error, the chief difficulty lies in the fact that the adsorbed quantity corresponding with the inhibiting concentration of p.aminobenzoic acid cannot be read from this curve, which was determined in the absence of sulfanilamide compounds. Determinations of concurrent adsorption of p.aminobenzoic acid and sulfanilamide side by side will be necessary here.

D. Experimental part.

Exp. I. Determination of the smallest concentration of sulfanilamide (or its derivatives) which is active.

B. coli was grown in a synthetic medium consisting of 1 g K_2HPO_4, 1 g $(NH_4)_2SO_4$ and 1 g Na pyruvate in 1000 ml tapwater. The medium was prepared in a more concentrated form and to 3 ml of this stock medium, 2 ml of solutions of the studied sulfanilamides in tapwater were added. With our usual technique (1) hourly determinations of the number of viable bacteria in these cultures were made. The growth curves,

derived from these data were plotted (f.i. fig. 2 and 3). In the ordinate the log of count is given per 28 mg of the culture. To obtain the log of count per ml log. 35 should be added. In fig. 2 and 3 instances of these experiments are given for sulfapyridine and methylsulfathiazol. For sulfanilamide the experiment has already been published (3).

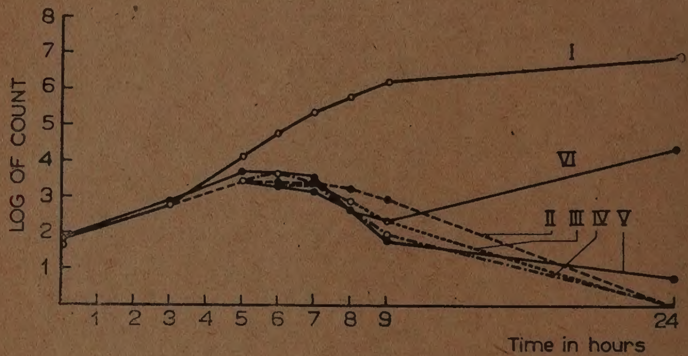


Fig. 2. The action of various concentrations of sulfapyridine on *B. coli*.

I Control. II, III, IV, V, VI with 2,1, 0.4, 0.2, 0.08 m.mol. sulfapyridine. The minima active concentration is 0.2 m.mol. (curve V).

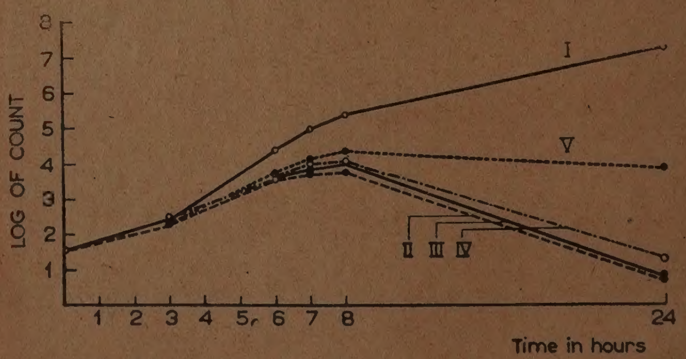


Fig. 3. The action of various concentrations of methylsulfathiazol on *B. coli*.

I Control. II, III, IV, V with 0.6, 0.2, 0.07, 0.03 m.mol. methylsulfathiazol. The minimal active concentration is 0.07 m.mol. (curve IV).

The smallest concentration, which within 24 hours reduced the number of viable bacteria below log.N = 1 was arbitrarily taken as a measure.

In most cases there was no difficulty in deciding upon the minimal active concentration. In some instances a culture with 0.1 % sulfanilamide was run simultaneously, thereby eliminating any possible differ-

ences between the various experiments, made at different times, with different batches of the medium etc. After finishing the determinations of the active concentrations of the studied compounds a further check was taken by testing the "active concentrations" of the six most important derivatives in one experiment. The obtained curves were essentially identical.

The minimal active concentrations for each drug are given in table I column a.

Exp. II. Determination of the adsorption of various sulfanilamide compounds by *B. coli.*

The method used in these experiments was described in full in no. VIII of this series (3).

B. coli was grown in glucose broth, the bacteria were centrifuged down, washed once with distilled water and resuspended in distilled water. The concentration of the suspension was estimated by centrifugation in capillary tubes and expressed as vol. %.

2 ml of a solution of sulfanilamide or one of the compounds were added to 2 ml bacterial suspension or to 2 ml distilled water. After centrifuging, the concentration of the drug in the supernate was determined (in duplicate) by diazotization and coupling to oxychinoline. The intensity of the orange-yellow colour of the diazo compound was estimated in a 3 cm cell in a Pulfrich Stuphometer with a filter S 50. The concentration of the drug in the supernate was derived from an extinction-concentration curve which was obtained with the control dilutions.

An instance of the determination of the adsorption isotherm $x/m = kc^{1/n}$ is given in table II. For p.aminobenzoic acid an analogous experiment was published in no. VIII of this series (3). All experiments were duplicated, generally with uniform results.

In table II an instance of an experiment is given for sulfapyridine with a 22 % suspension of bacteria. All concentrations are given in millimoles per litre; C = concentration in the control. C_1 = equilibrium concentration after adsorption (*i.e.* of the supernate).

The adsorbed quantity is obtained as a difference in concentration $C—C_1$. As the volume of the bacteria after dilution is 11 %, the adsorbed quantity $x/m = 11/100 \times (C—C_1)$ m.mol. per litre of bacteria. The concentration in the bacteria apart from the adsorbed quantity evidently is C_1.

Table II

C	C_1	$C—C_1$	x/m	log C_1	log x/m
0.400	0.382	0.018	0.162	0.58–1	0.20–1
0.200	0.192	0.008	0.072	0.28–1	0.86–2
0.100	0.096	0.004	0.036	0.98–2	0.56–2
0.050	0.048	0.002	0.018	0.68–2	0.26–2

We wish to draw attention to the fact, that for the curves V and VI and to a lesser degree also for curve IV (fig. 1), the adsorbed quantity is only just above the experimental error. And though the order of sequence of these three drugs was established beyond doubt, the absolute position of the curves should be regarded with some caution.

Exp. III. Determination of the concentration of p.aminobenzoic acid, which completely inhibits the minimal active concentration of a sulfanilamide compound.

B. coli was cultured in the synthetic medium already mentioned (Exp. I) and the influence was studied of various concentrations of p.aminobenzoic acid on the action of the sulfanilamide-compounds under discussion in their minimal active concentrations. The viable count,

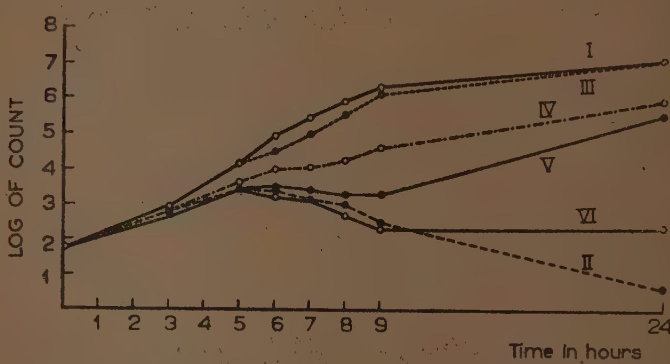


Fig. 4. The inhibition of sulfapyridine action by p. aminobenzoic acid (*B. coli*).

I Control. II with 0.2 m.mol. sulfapyridine. III, IV, V, VI with 0.2 m.mol. sulfapyridine and 0.005, 0.0017, 0.0005, 0.0001 m.mol. p. aminobenzoic acid respectively. Complete inhibition with 0.005 m.mol. p. aminobenzoic acid (curve III).

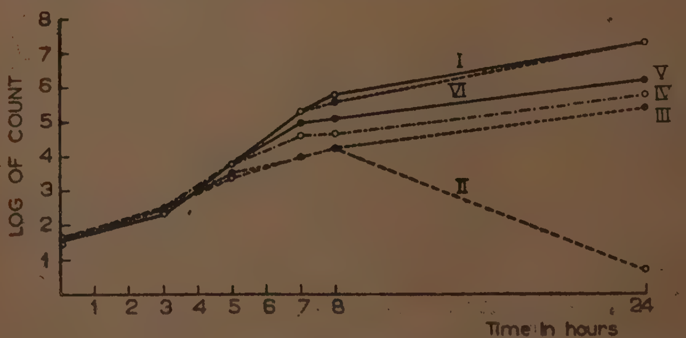


Fig. 5. The inhibition of 2-sulfanilamido-4-methylpyrimidine by p.aminobenzoic acid (*B. coli*).

I Control. II with 0.15 m.mol. 2-sulfanilamido-4-methylpyrimidine. III, IV, V, VI with 0.15 m.mol. 2-sulfanilamido-4-methylpyrimidine and 0.0001, 0.0005, 0.0017, 0.005 m.mol. p. aminobenzoic acid. Complete inhibition with 0.005 m.mol. p. aminobenzoic acid (curve VI).

made with our usual technique was used as a measure. Some instances of the resulting growth curves are given in the figs. 4 and 5 (for sulfapyridine and 2-sulfanilamido-4-methyl-pyrimidine respectively). For sulfanilamide an analogous experiment has already been published in no. VIII of this series (cf. fig. 4, p. 149 (3)). The inhibiting concentration of p.aminobenzoic acid derived from these growth curves is given in table I, column d.

p.Aminobenzoic acid in a concentration of 0.005 m.mol. per litre is able to re-establish growth completely in every case. In some cases (f.i. no. VIII of this series, fig. 4) the growth curve with 0.005 m.mol. p.aminobenzoic acid was not quite identical with the control.

Considering this and the experimental error of the determination of the inhibiting concentration it is possible that this concentration should be estimated slightly higher than 0.005 m.mol. It is quite evident, however, that the inhibiting concentrations were equal in all cases.

Summary.

The better activity (in vitro) of various sulfanilamide compounds as compared with sulfanilamide itself is only quantitative, *i.e.*, an equal activity is obtained with lower concentrations. It is shown, that the activity of the studied drugs is so narrowly related to their adsorption in the bacteria (*B. coli*), that probably the varying activity of the studied compounds is due to differences in adsorbability. For different drugs the adsorbed amount was equal for concentrations with equal activity.

The concentration of p.aminobenzoic acid which re-establishes growth — in cultures containing the studied compounds in concentrations of equal activity — was equal in all cases. This fact corroborates the hypothesis, that activity and adsorption are correlated and shows, that the mechanism of action (in vitro) is the same in all cases.

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VI-ANTIGEN IN *B. COLI* AND VI-AGGLUTININ

by

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(Received August 22, 1943).

The serological diagnostics of the *Salmonella* infections have been much improved owing to our knowledge of the antigenic structure of this bacteria, which has been laid down in the KAUFFMANN–WHITE scheme. For the differentiation of the *Salmonella* group many O- and H-antigens are used, as well as the Vi-antigen discovered by FELIX and PITT (10) with *S. typhi*. Besides in *S. typhi* this latter antigen is only found in *S. paratyphi* C (KAUFFMANN (14)), *S. ballerup* (KAUFFMANN and MØLLER (17)) and *B. coli* (KAUFFMANN (15)).

As Vi-antigen containing *S. typhi* are especially derived from chronic carriers of bacteria (CRAIGIE and BRANDON (4), WELCH and MICKLE (25), OGONUKI (20), FELIX (9), RAUSS (23) a.o.) various workers tried to trace these carriers by proving the presence of the Vi-agglutinin corresponding with the Vi-antigen in their bloodserum. According to information given by FELIX, ELIOT (6), BHATNAGER (2), PIJPER and CROCKER (21), GIOVANARDI (12), FAARUP (7) and RAMMLER (22), this method leads to very good results.

DAVIS (5) examined the serum of 656 natives from South-Rhodesia and he found the Vi-agglutinin in 49 cases (= 7.5 %) with a titer from 1 : 5 to 1 : 12.5. FAARUP found the Vi-agglutinin in 15 out of 190 normal sera, *i.e.*, 8 %, with titers varying from 1 : 10 to 1 : 50, whilst RAMMLER succeeded in showing the presence of the latter in 8 out of 188 normal sera (4 %) with a titer of 1 : 5.

Investigating the presence of *Salmonella* antigens in *B. coli* we (1) found that the Vi-antigen occurred in 7—8 % of the cases wherein *B. coli* had been derived from the intestines of healthy persons. In connection with the problem of the genesis of the natural agglutinins we thought it of interest to investigate, whether there could be found any connection between the Vi-antigen in *B. coli* from the intestines and the Vi-agglutinin in the blood of healthy individuals; in this way we meant to answer the question whether this natural Vi-agglutinin is the consequence of an infection by the pathogene *S. typhi*, or that it might be caused by the light attacking action that the commensal *B. coli* can maintain within the host. There are, as known, two theories about the origin of the natural agglutinins; the first regards them as a token of physiological ripening (serogenesis of H. and L. HIRSCHFELD) and the second attributes them to the presence of an other bacterium with similar antigens (for a good review of this problem we refer to REURINK (24)).

For this purpose we investigated 600 sera having been sent to us for

examination by means of the WASSERMANN-reaction and proved negative for the latter as to the occurrence of the Vi-agglutinin.

As ficker we used a suspension in formaldehyd of the Vi-I strain of BHATNAGER (11). This strain exclusively possesses the Vi-antigen, no O- or H-antigen (the use of a strain of *B. coli* with Vi-antigen without other *Salmonella* antigens gave about the same results). The agglutination was read in the dilutions 1 : 5, 1 : 10, 1 : 20, 1 : 40 etc. after the sera had been kept for two hours at 37° C. and at room temperature during 20 hours.

54 out of these 600 sera proved to contain Vi-agglutinin, *viz.*, 30 with a titer of 1 : 5, 19 with a titer of 1 : 10, 4 with a titer of 1 : 20 and 1 with a titer of 1 : 40. In these cases we were supplied with faeces from the individuals concerned in order to be able to investigate whether these samples contained *B. coli* with Vi-antigen. Moreover, as a control, we also examined faeces originating from healthy persons, who had no Vi-agglutinin in their serum. In some cases, in which the Vi-agglutinin was found in the serum, an infection with typhoid or paratyphoid appeared to have occurred in the anamnesis or an immunization against such an infection. Of course such cases were left out of account.

For comparison with *B. coli* from the faeces there remained 32 sera with Vi-agglutinin and 26 sera without Vi-agglutinin. We arrived at the following results:

Vi-agglutinin	in serum	+,	Vi-antigen	in	*B. coli*	+ : 14 ×
,,	,,	,, +,	,,	,,	,,	— : 18 ×
,,	,,	,, —,	,,	,,	,,	+ : 10 ×
,,	,,	,, —,	,,	,,	,,	— : 16 ×

Apparently we have not succeeded in proving a connection between the Vi-agglutinin in the serum and the Vi-antigen in *B. coli* from the intestines, for this antigen was found about as many times together with the agglutinin as in its absence and conversely.

When these experiments were effected, the L-antigen of *B. coli* had not yet been described by KAUFFMANN (16). Whenever this antigen is found, *B. coli* shows the phenomenon of O-inagglutinability, but it is not stated, whether also the Vi-agglutinability disappears. Yet this L-antigen can have had little if any influence on the outcome of our experiments, as it is principally found by means of *B. coli* isolated from urine during infections of the urinary ducts, from pus, gall, blood etc., and seems to occur seldom in *B. coli* isolated from sound intestines (KAUFFMANN and PERCH (18)). Nevertheless the possibility of a connection between the Vi-agglutinin and the Vi-antigen of *B. coli* is not yet wholly excluded by the result of our experiments, for examinations made by MIKKELSEN (19), by KAUFFMANN and PERCH (18), by BOIVIN, CORRE and LEHOULT (3), and by ourselves (1), have made it known, that in the intestines different strains of *B. coli* may occur together and that some strains may disappear, which may reappear later on.

As we were only in a position to examine for each case a single sample of faeces, we could only make a so-called snapshot, which needs not necessarily be a true picture of the real situation in the intestinal canal and so the possibility exists that strains with Vi-antigen have been present without having been demonstrated by us.

As regards the absence of the Vi-agglutinin in those cases in which *B. coli* with Vi-antigen were found, we mean to suggest that not every

individual needs to react upon the presence of an antigen by forming an agglutinin and moreover it is possible, that the antigen found had not yet lead to the formation of an agglutinin, the presence in the body having not lasted long enough for this.

So further experiments will be necessary to solve this problem.

At first we had the intention to examine the problem also experimentally in the same way as INGALLS (13) did, by feeding young rabbits with *B. coli* containing Vi-antigen, and by examining whether the Vi-agglutinin would appear in the serum after some lapse of time. Present conditions, however, have prevented us to accomplish these experiments with animals, but we hope to be in a position to do so as yet in due course.

Summary.

The Vi-agglutinin in normal serum occurs in 4—8 %, the Vi-antigen in *B. coli* out of faeces of healthy persons in 7—8 % of the cases. The authors could not, however, prove a connection between these two phenomena.

The authors present some reasons why such a connection need not to be considered as absolutely impossible after all.

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THE CLASSIFICATION OF THE PLAGUE-BACILLUS

by

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In the fifth edition of BERGEY's Manual of Determinative Bacteriology (1939) the genus *Pasteurella* — together with the genera *Brucella* and *Hemophilus* — has been placed in the family *Parvobacteriaceae*, a classification recently proposed by RAHN (4).

Consequently the plague-bacillus (*Pasteurella pestis*) and the bacillus of pseudotuberculosis rodentium (*Pasteurella rodentium*), although both of moderate size, are automatically included in a family which is characterised, as has been postulated by its author, by the exceptionally small size of its members.

It is therefore apparent that the classification of the plague-bacillus and of the rodentium bacillus, which also previously was far from satisfactory, urgently needs revision.

The relation between the plague-bacillus and the type-species *Pasteurella avicida* is by no means close. They differ not only by size, but by their biochemical properties, antigenic structure and the character of their agar colonies as well (See Table).

The main reason that they have been grouped in the same genus seems to be that in stained preparations the ends of the rods take the dye more markedly than the central part, especially in bacteria derived directly from the body. However it must be recognised that occasionally this character is also present in other genera and that even in this bipolar staining the plague-bacillus differs from the typical *Pasteurella*. In the latter the bipolarity is far more regular than in the former; very often smears from infected material contain plague-bacilli which did not take the dye exactly at the ends, but more or less obliquely or even at one side only (securety-pin).

RAHN (4) himself recognises the exceptional position of the „large" plague-bacillus among the *Parvobacteriaceae*, stating that „the relationship of this group must be considered only tentative".

These are the motives for my proposition to classify the plague-bacillus, together with the nearly related rodentium bacillus, in a new genus: *Yersinia* [1]) (*Y. pestis*, *Y. rodentium*) and to place this genus, with other unclassifiable genera into the family of the *Bacteriaceae*.

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[1]) A. J. E. YERSIN (1863–1943) discovered the plague-bacillus in 1894.

Table

	Plague-bacillus (*Yersinia pestis*)	*Pasteurella avicida*
Average length	1.5—1.8 μ (ALBRECHT and GHON cf. (2))	0.15—0.25 μ (HUTYRA cf. (3))
Growth in plain commercial meat extract agar	abundant	scanty
Colony on agar	raised opaque yellow centre and flat, clear periphery with crenellated margins	smooth, translucent, even-edged
Growth on potato	+	—
Growth in yeast-water (cf. (1))	+	—
Growth in the presence of bile salts	+	—
Rhamnose	+	—
Saccharose	—	+
Sorbitol	—	+
Indol	—	+
H_2S	—	+
Antigenic structure	No cross-agglutination	
Plague-phage (cf. (1))	+	—

I am not quite sure whether the name *Yersinia* has ever been proposed; I did not succeed, however, to retrace it in literature.

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A PHENOMENON RESEMBLING ANAPHYLACTIC SHOCK AFTER TREATMENT WITH SULPHAPYRIDINE

by

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In two earlier communications (4,6) we have offered arguments for the conception that the fatal course of cases of pneumonia treated with sulphapyridine might have been caused by general anaphylactic reaction and that the necroses in lung tissue we could note in some of such cases might be due to a local anaphylactic reaction. This conception is based on the results we have arrived at in experiments on rabbits with filtrates of inflammated lungs of rabbit. In fact a filtrate prepared out of inflammated lungs of rabbit having been treated with sulphapyridine could induce an anaphylactic shock in a notably smaller amount than when no sulphapyridine had been administered. So in rabbits, which had outlived a pneumonia, sulphapyridine lung filtrate could give rise to shock in an amount at least ten times smaller than the amount needed when no treatment with sulphapyridine had taken place. So we could conceive that patients suffering from pneumonia might also be sensibilized to one or more antigens present in the inflammated lungs and that the treatment with sulphapyridine might further the occurrence of anaphylaxis. In our formerly described experiments on animals we had not succeeded in inducing a chemospecific anaphylaxis with sulphapyridine. WERTH (5), however, has proved that sulphapyridine without the addition of protein can exercise anaphylactic action on guinea pigs. A guinea pig, intraperitoneally sensibilized with sulphapyridine, shows after three weeks a positive DALE test.

Some further facts induced us to take up again the investigation regarding an existence of chemospecific anaphylaxis to sulphapyridine. These facts concern two patients who had died at the Surgical Clinic at Leiden (Prof. SUERMONDT), respectively nine and ten days after a succesful surgical intervention.

The first patient, a man of 44 years, had been suffering for a few years from gastric ulcer. Notwithstanding a conservative treatment his complaints

had not decreased. Blood had been repeatedly detected in the stools. Therefore an operation (gastric resection) was performed.

The second patient, a man of 42 years, had been admitted on account of a perforation of the stomach. In the previous year he had shown symptoms of gastric ulcer. Besides this he had always been in good health.

After the operation sulphapyridine had been administered to both patients (1—4 g intramuscularly) during respectively eight and five days in view of the possibility of postoperative pneumonia. Two or three days after the operation the general condition of both patients was excellent. Then both developed fever. A few days later the first patient showed cyanosis. During the following five days the body temperature kept on increasing. Then he collapsed and died on the same day. The second patient expectorated a purulent sputum at the seventh day after the operation. Two days later also his general condition deteriorated rather suddenly and he died on the next day. The administration of sulphapyridine had been brought to a close three days before his death, whilst the first patient had received this drug up till the day before his death.

Post mortem examination revealed in both cases hemorrhagic serous cellular bronchopneumonia in the caudal parts of the lungs. Hyperaemia of the lungs and of the abdominal viscera occurred. The heart did not show any particular abnormality. In the operation region no definite alterations could be noted. The peritoneum was smooth and glossy.

The fact that on the one hand before the operation both patients had been in good condition and on the other hand that the operation in both cases had been very successful, which latter fact was confirmed by the obduction, made us doubt whether the pathological findings: serous cellular bronchopneumonia, could actually account for the death. In this connection the surgeon (H. G. RUHAAK) put in the question whether the prophylactic administration of sulphapyridine might not in these cases have affected the course of the disease unfavourably.

As in our opinion in pneumonia anaphylaxis can be furthered by the administration of sulphapyridine the question arose, whether in the cases at hand the reverse might not have taken place. In other words: might this be a case of sensibilization by sulphapyridine and is it possible that the antigens which are formed in the course of pneumonia appearing after the sensibilization, might have furthered the arising of an anaphylactic reaction? In order to be able to answer this question, we tried again to induce a chemospecific anaphylaxis in guinea pigs with sulphapyridine. In this experiment the arrangement such as it was followed in our former experiments was somewhat altered. At the same time we investigated whether the filtrate of inflammated human lung tissue might exercise a shock on guinea pigs having been sensibilized with sulphapyridine and whether the filtrate might further the arising of chemospecific anaphylaxis.

To man sulphapyridine is administered per os as well as parenterally during several days at a stretch. According to the present-day conceptions about the arising of anaphylaxis an anaphylactic reaction might be expected only when the sensibilization had been induced by a small dose of anaphylactogen administered parenterally, followed after at least ten days by a re-injection of the same

anaphylactogen directly into the bloodvessel in a dose several times the strenght of the initial one. Repeated administrations such as were performed on our patients would render a desensibilization rather more probable than a sensibilization. Moreover the sulphapyridine had been injected during eight and five days; so the incubation period had been rather short for an anaphylactic reaction. We have tried, however, to sensibilize guinea pigs with sulphapyridine by one or more intramuscular injections of 50 mg (on successive days) or by doses per os of 250 and 5C0 mg. The anaphylactic injection was administered intracardially with 1CC mg of sulphapyridine at various times after the first administration.

Table I

Guinea pig	sensibilization		anaphylactic injection		shock
	dose of sulphapyridine	mode of administration	dose of sulphapyridine	days after the first injection	
159	1 × 50 mg	intramuscular	100 mg	1	—
160	2 × 50 ,,	,,	,,	3	—
162	3 × 50 ,,	,,	,,	4	—
168	1 × 50 ,,	,,	,,	4	—
161	4 × 50 ,,	,,	,,	5	+
169	2 × 50 ,,	,,	,,	5	+
170	2 × 50 ,,	,,	,,	7	+
164	5 × 50 ,,	,,	,,	8	—
163	5 × 50 ,,	,,	,,	10	—
165	6 × 50 ,,	,,	,,	10	+
171	2 × 50 ,,	,,	,,	10	+
166	7 × 50 ,,	,,	,,	11	—
167	7 × 50 ,,	,,	,,	12	—
172	2 × 50 ,,	,,	,,	12	+
153	5 × 50 ,,	,,	,,	14	+
154	5 × 50 ,,	,,	,,	14	+
264	1 × 500 ,,	per os	,,	6 and 14	—
286	4 × 250 ,,	,,	,,	8	—
275	1 × 500 ,,	,,	,,	13	+
265	1 × 250 ,,	,,	,,	14	+
280	4 × 250 ,,	,,	,,	14	+
283	4 × 250 ,,	,,	,,	14	+
276	1 × 250 ,,	,,	,,	16	—
288	1 × 500 ,,	,,	,,	21	—
289	1 × 250 ,,	,,	,,	21	—
291	1 × 500 ,,	,,	,,	21	—

As is shown in table I sensibilization of guinea pigs with sulphapyridine would actually be possible per os as well as intramuscularly by repeated administration. The intracardial anaphylactic injection gives rise to the symptoms of shock in 4C—50% of the animals, none of which cases, however, are fatal. A deadly shock, however, can be provoked by means of a re-injection with a mixture of sulphapyridine and lung filtrate.

Table II

Guinea pig	sensibilization		anaphylactic injection		days after the first injection	shock
	dose of sulphapyridine	mode of administration	dose of sulphapyridine	dose of lung filtrate		
146	40 mg	intramusc.	200 mg	1 cc	14	++
149	20 ,,	,,	200 ,,	1 ,,	14	—
152	40 ,,	,,	100 ,,	1 ,,	14	++
155	5 × 50 ,,	,,	100 ,,	1 ,,	14	+
156	5 × 50 ,,	,,	100 ,,	1 ,,	14	+
150	40 ,,	,,		2 ,,	14	—
151	60 ,,	,,		3 ,,	14	—
157	5 × 50 ,,	,,		2 ,,	14	—
158	5 × 50 ,,	,,		2 ,,	14	—
281	4 × 250 ,,	per os		2 ,,	14	—

++ deadly shock.
+ non-deadly shock.

The lung filtrate has been prepared out of inflammated human lung tissue. For this purpose this matter is cut to pieces and ground finely; it is subsequently suspended in 10 parts of a physiological saline solution (0.9% NaCl), shaken for two hours and finally filtered of through a double gauze and through a Seitz E.K. filter.

The intracardial injection of the lung filtrate on guinea pigs sensibilized with sulphapyridine and the injection of sulphapyridine lung filtrate in non-sensibilized guinea pigs does not give rise to any reaction.

The actual possibility to sensibilize a guinea pig per os seems to disagree with the conception about the arising of protein anaphylaxis. Though in exceptional cases it is possible for a protein to pass the intestinal wall, the proteins are generally broken down in the intestinal tract, thus losing their antigenic character and sensibilizing action. Sulphapyridine however is not a protein and so it can pass the intestinal wall in an unchanged condition. According to KIMMIG and WESELMANN (3) the sulphanilamides are haptens, which only attain an antigenic character by their linkage to serum protein. We can imagine that by these means sulphapyridine notwithstanding the peroral administration may appear in the blood and may exert an antigenic action after its linkage to the serum or organ proteins and may sensibilize the organism in that way. Contrary to the anaphylactic shock in such experiments shock symptoms can be induced as early as five days after the initial injection, whilst a desensibilization by the repeated intramuscular and peroral doses has not been induced. Moreover Table I shows that the sensibilized animals remain sensitive to the shocklike symptoms during a merely short period, for this phenomenon only appears in guinea pigs that have been sensibilized 5 or 14 days earlier. Whereas after a lapse of 21 days

the phenomenon can no longer be induced. The protein anaphylaxis, on the other hand, remains for life. The anaphylactic reaction is regarded as an antigenic-antibody reaction. It cannot be conceived that in merely 5 days antibodies would have been formed. So we are of the opinion that these arguments can be advanced against the conception that the symptom described by us might be a mere anaphylactic reaction.

How can we account for it, however, if not in this way? The doses of the sulphapyridine injected are not toxic, nor is the toxic dose reached by cumulation. If we inject a guinea pig intracardially with 400 mg of sulphapyridine the animal shows, not immediately but after a short lapse of time, convulsions which may last for one hour and longer. The shock symptom, however, only appears in sensibilized guinea pigs immediately after the intracardial re-injection. Clinically it cannot be distinguished from the anaphylactic shock and it lasts for five minutes at most. At autopsy the guinea pigs (after the injection with sulphapyridine lung filtrate, Table II) show hyperaemia of the abdominal organs, emphysematous changes of the lungs and hemorrhagiae are found under the pleura pulmonalis. Like symptoms, among which the hemorrhagic reaction, could also be noted in the patients. A similar reaction is seen in the SANARELLI–SHWARTZMAN phenomenon. As it is known a rabbit will die with the symptoms of anaphylactic shock after the intravenous injection of colifiltrate, provided that the animal has been injected 24 hours earlier with a subletal dose of cholera vibrios (phenomenon of SANARELLI) and that a hemorrhagic necrotic reaction will appear on a rabbit after the intravenous injection of a filtrate of *B. coli*, *B. typhosus* or other bacteria, provided that such a filtrate has been injected intracutaneously 24 hours earlier (phenomenon of SHWARTZMAN).

With chemical substances as well a phenomenon can be excited which shows some analogy to the former phenomena, *viz.*, the phenomenon of GLAUBACH (2). Here a deadly shock can be induced in rats by means of the subcutaneous injection of a harmless dose of papaverine 2—3 hours after the peroral administration of a harmless dose of sulphapyridine (DUREL and RATNER (1)).

Though the shock phenomenon observed by us can appear no earlier than after five days and the phenomena of SANARELLI–SHWARTZMAN and of GLAUBACH appear already after respectively 24 and 2 hours, it is yet possible that they may be of a same nature. In each of these phenomena after a preceding sensibilization a shock will appear which cannot be distinguished from an anaphylactic shock. Whilst the anaphylactic reaction, however, is strictly specific, *i.e.*, only appears when the same substance is used in the reinjection as in the initial sensibilizing injection, the phenomena of SANARELLI–SHWARTZMAN and of GLAUBACH are induced by a substance other than the initial one. The phenomenon described by us is essentially specific to sulphapyridine but it is intensified moreover by an unspecific component, *viz.*, the lung filtrate. The

results of our experiments might point to the possibility of sulphapyridine exerting a sensibilizing action on man as well and we can imagine that in case a pneumonia develops notwithstanding the prophylactic treatment with sulphapyridine shock phenomena are more likely to appear than without the occurrence of pneumonia.

If this is true death can be only accounted for by shock. This would agree with the fact that in the two fatal cases described the pneumonic alterations found cannot sufficiently account for the death. Already at the beginning of pneumonia, however, strange proteins (organ- and species-specific ones) which can pass into the blood, are formed in the lungs and these may be put on a level with the lung filtrate used in the experiments on guinea pigs.

Summary.

Two patients on which a successful operation of the stomach had been performed developed fever some days after the operation, notwithstanding a prophylactic treatment with sulphapyridine and both of them died rather suddenly respectively 9 and 10 days after the operation. On obduction in both cases hemorrhagic serous-cellular bronchopneumonia were found in the caudal parts of the lungs, all the organs were very hyperaemious and the heart did not show any alteration. To account for the fatal course the possibility of chemospecific anaphylaxis to sulphapyridine has been considered. We succeeded in inducing in guinea pigs by means of sulphapyridine a shock, which, however, did not result in death. Such a shock could be induced as early as five days after sensibilization. The adding of the filtrate of inflammated lung tissue resulted in a deadly shock. In this connection the surmisal was made that also in the patients a sensibilization by sulphapyridine had occurred and that the pneumonia, which as such could not sufficiently account for the death, has furthered the arising of shock. We have pointed to the various facts which disagree with the identification of the phenomenon observed with an anaphylactic shock. We mention, however, that there is a certain agreement with the phenomenon of SANARELLI–SHARTZMAN and that of GLAUBACH.

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ÉPIDÉMIE SURVENUE PARMI DES SOURIS BLANCHES, À LA SUITE D'UNE INFECTION PAR LE *CORYNEBACTERIUM PSEUDOTUBERCULOSIS MURIUM*

par

M. F. POLAK

(Reçu le 30 Septembre 1943).

Parmi les épidémies qui peuvent survenir au laboratoire chez les souris blanches, quelques-unes sont caractérisées par une formation de tubercules en divers organes et comprises par le nom de „pseudotuberculose". Pour autant qu'elles appartiennent au domaine bactériologique, les espèces suivantes sont principalement indiquées comme agents de cette pseudotuberculose: *Bact. pseudotuberculosis rodentium* (*Pasteurella pseudotubercolosis*), différentes *Salmonellae* et le *Corynebacterium pseudotuberculosis murium.* REZEK et LAUDA (4) communiquent, dans leur discussion de la pseudotuberculose du foie des animaux de laboratoire, caractérisée par des nécroses en foyers à réaction inflammatoire, que *Corynebacterium pseudotuberculosis murium* ne provoquerait jamais d'anomalies dans cet organe. Ceci n'est pas en complet accord avec les données bibliographiques; or, ayant pu observer parmi les souris blanches de l'„Instituut voor Tropische Hygiëne" une épidémie provoquée par cette bactérie et dans laquelle, seul, le foie était atteint, nous donnons ici la description de nos observations.

Au cours de l'an dernier, lors de nos expériences effectuées sur des souris blanches pour des recherches sur la fièvre jaune, il surgit des difficultés par suite d'une infection introduite par deux fois dans notre matérial avec un envoi de souris livrées par un même fournisseur. Peu après l'arrivée des animaux au laboratoire, il survint des cas de morts „spontanées" enlevant plusieurs souris par jour, de sorte que quelques semaines plus tard, il restait fort peu d'animaux d'un groupe de 50 souris. Le syndrome clinique était peu caractéristique: les animaux perdaient leur activité, leur pelage devenait hirsute, ils se tenaient immobiles, le dos en boule et leur défécation était fréquemment de consistance liquide. L'autopsie, toutefois, révéla que le foie présentait toujours un aspect caractéristique: il était parsemé de petits foyers d'un blanc grisâtre,

de 2 mm de diamètre au plus, pas très ronds et mal délimités d'avec le tissu normal environnant. La rate était grosse, mais par ailleurs normale. Des cultures, faites à l'aide de ces foies, donnèrent un micro-organisme présentant une parenté très étroite avec le „*Bacillus*" *pseudotubercolosis murium* que KUTSCHER (3) isola en 1894 de poumons de souris infectées et que l'on compte maintenant parmi les diphtéroïdes sous le nom de *Corynebacterium pseudotuberculosis murium.* Il a été fait un examen microscopique du foie de l'une de nos souris: les petits foyers consistaient en infiltrations de polynucléaires avec quelques lymphocytes et, souvent, avec nécrose centrale du tissu hépatique; les limites des cellules étaient vagues, les noyaux pyknotiques et fragmentés. Le Gram révéla que les infiltrations étaient pleins de bactéries: bâtonnets minces et parfois un peu arqués, positifs au Gram et de longueur variée, atteignant au maximum 3 μ. Ils étaient irrégulièrement colorés et montraient parfois une coloration des granules et des pôles; ils ressemblaient fortement à des bactéries de diphtérie. Les micro-organismes se bornaient pour ainsi dire au tissu anormal, bien qu'il y eût parfois encore quelques groupes dans le territoire avoisinant.

L'ensemencement de foyers de foie sur le sang gélosé nous donna plusieurs fois une culture pure de bactéries de ce genre. Or, maintenant, le Gram les montrait plus courtes (1—2 μ) et de forme plus lourde souvent amassées en formation V. Colorées selon Neisser, elles montraient, quelquefois rarement et quelquefois en abondance, une coloration polaire, qui, d'ailleurs, n'impliquait pas toujours les deux pôles. Les grains ne faisaient pour ainsi dire jamais saillie à l'extérieur du bâtonnet et ils étaient quelquefois très gros.

Sur la gélose, les bactéries avaient formé, en 3 fois 24 heures (par 37° C.), de petites colonies rondes, d'un blanc jaunâtre, quelque peu transparantes, à surface convexe et finement granulée avec un bord très net. En 6 fois 24 heures, leur diamètre avait atteint 0.5 à 1 mm. Sur le sang gélosé et dans le sérum coagulé, la croissance avait été beaucoup plus rapide. La gélatine en piqûre ne montrait aucune croissance après 24 heures; après 72 heures et plus, on y voyait un trait d'ensemencement grisâtre non branché. Le bouillon était trouble après 1 jour, avec un léger dépôt tourbillonnant rapidement au secouage; nous n'avons pas observé de formation cristalline. Les micro-organismes étaient toujours immobiles.

Le comportement biochimique était celui-ci: pas de formation d'indol, pas de modification du lait tournesolé et du milieu d'Oldekopp, fermentation de glucose, maltose et saccharose causant une acidification sans gaz, tandis que lactose, mannitol, glycérol et adonitol ne sont pas détruits. La gélatine n'est pas liquéfiée, le lait ne coagule pas; il n'y a ni hémolyse, ni hémodigestion.

Ces caractéristiques établies, il nous semble pouvoir dire que la bactérie que nous avons isolée est un diphtéroïde, identique ou

étroitement apparenté au *Corynebacterium pseudotuberculosis murium* que KUTSCHER décrivit le premier. D'autres auteurs ont, eux-aussi, isolé des bactéries de ce genre chez des souris ou des rats (REED (5), BONGERT (1), GUNDEL, GYÖRGY et PAGEL (2)); ils mentionnent de petites différences dans la morphologie et le mode de croissance, le syndrome provoqué chez les souris n'est pas, lui non plus, toujours absolument le même. KUTSCHER vit une seule fois, chez l'une de ses souris d'expérience, de petits foyers hépatiques; l'inoculation sous-cutanée de sa bactérie n'amenait point la mort de ses souris. Par contre, BONGERT observa plusieurs fois des anomalies dans le foie et, de plus, dans les reins et la rate; il vit souvent aussi du gonflement des glandes lymphatiques. L'inoculation sous-cutanée était mortelle. REED n'a jamais observé de foyers hépatiques lors de ses expériences d'infection. Toutefois, lors d'infection spontanée, la localisation pulmonaire est très générale et le poumon présente de petits tubercules à centre caséeux. Ceci, pourtant, n'a jamais été le cas dans nos expériences; les petits foyers hépatiques étaient les seules anomalies visibles.

Afin de prouver que le corynebacterium isolé était l'agent de l'infection attaquant nos souris, nous avons pratiqué des expériences d'infection chez des souris blanches, saines, d'autre provenance.

L'injection sous-cutanée d'un 0.5 cc de suspension de bactéries (1/20 du tube de sérum coagulé incliné) provoqua la mort de 3 souris en, resp. 7, 11, et 14 jours. Il y avait, à la place de l'injection, une grande infiltration déjà plus ou moins ramollie. Le pus donna une culture pure de *Corynebacterium pseudotuberculosis murium.*

L'inoculation intrapéritonéale amenait la mort de la souris dans les 3 à 12 jours (dépendant, entre autre, de la dose). On découvrit, dans tout l'abdomen des animaux morts en peu de temps, une couche fibrineuse sur le péritoine viscéral et pariétal. Il se développait chez ceux ayant vécu plus longtemps de petits foyers hépatiques. Nous n'avons pas observé d'hypertrophie des glandes lymphatiques du mésentère. Le corynebactérium injecté a toujours pu être cultivé, à l'état pur, à l'aide du foie.

Du moment que nous supposions que l'infection naturelle se produit par la bouche, nous avons tenté d'infecter un certain nombre de souris au moyen des aliments et traité, à cet effet, 7 animaux. L'un mourut après 1 jour, un second survécut et fut sacrifié un mois après; il ne montrait aucune anomalie. Parmi les 5 autres, 3 moururent en, resp., 9, 13 et 14 jours, présentant des anomalies pulmonaires et 2, en, resp. 9 et 22 jours, présentant des anomalies pulmonaires et les petits foyers hépatiques en question. Peu avant leur mort, tous ces animaux avaient une dyspnée manifeste. Les symptômes pulmonaires nous surprirent; les poumons étaient remplis de tubercules blancs-roses, assez nettement délimités du tissu pulmonaire hyperémique, mais

normal par ailleurs; leur forme était irrégulière et leur diamètre mesurait 3—4 mm. environ. Les tubercules étaient nettement proéminents à l'extérieur de la surface du poumon; la plèvre était adhérente, mais toujours sans exsudat. Les glandes lymphatiques de la poitrine et du cou n'étaient pas hypertrophiées. Nous avons cultivé plusieurs fois, à l'état pur, le *Corynebacterium pseudotuberculosis murium* à l'aide des foyers pulmonaires.

Ces tubercules des poumons concordent avec les anomalies que, d'après la bibliographie, on trouve régulièrement lors de l'infection naturelle par la bactérie de Kutscher; mais, dans notre cas, ils ne se sont manifestés qu'après une infection artificielle, dont le but était d'imiter l'infection naturelle. Il est possible que, lors de contamination orale moins massive en soi que celle appliquée dans nos expériences, les foyers hépatiques eussent été les seuls symptômes.

Kutscher n'a point réussi dans ses expériences à infecter des souris par la bouche; la vaporisation régulièrement pratiquée des suspensions de bactéries a toutefois provoqué les anomalies pulmonaires caractéristiques. Topley et Wilson (6) n'ont observé les anomalies pulmonaires que de temps à autre, après l'infection artificielle, et en bien moindre mesure que lors d'infection naturelle. Quant à nous, c'est justement dans ce dernier cas que nous n'avons jamais vu de lésions pulmonaires, tandis que la contamination artificielle orale les provoquait.

Nous avons examiné plusieurs fois, au microscope, le foie et les poumons de souris infectées artificiellement. Le foie montrait des anomalies concordantes avec celles citées plus haut et il y avait, dans les poumons, des foyers étendus de pneumonie purulente; les alvéoles présentaient une forte infiltration de polynucléaires, tandis qu'on voyait en même temps des gros amas de bactéries ressemblant nettement à des corynebactériums, tant avec le Gram qu'avec le bleu de méthylène de Loeffler. D'autres parties du poumon montraient de grands hématomes.

L'injection intrapéritonéale de filtrat de cultures faites dans du bouillon n'amenait pas la mort des souris; à l'encontre d'autres auteurs, nous n'avons donc aucune raison d'admettre ici la formation d'une exotoxine. Mentionnons ensuite que l'ensemencement de sang cardiaque de souris infectées a eu régulièrement un cours négatif.

Nous pensons pouvoir conclure, en résumé, que le corynebactérium que nous avons isolé, est l'agent de l'épidémie constatée chez nos souris blanches. Cette bactérie appartient à un groupe prochement apparenté de diphtéroïdes dont Kutscher fut le premier à décrire un réprésentant. Ces organismes provoquent, chez la souris, des foyers d'infiltration nécrotiques, dont la dispersion dans l'organisme varie cependant.

La lutte contre la maladie comprend peut-être, outre la prise de mesures générales (isolation, sacrifice des animaux malades

et, éventuellement, des animaux suspects), une modification de l'alimentation. GUNDEL et ses collaborateurs communiquent que leurs rats n'eurent une pneumonie que s'ils souffraient d'une déficience en vitamine H. Cette substance a été active aussi thérapeutiquement.

Résumé.

Une épidémie ayant atteint des souris blanches de laboratoire, a présenté le syndrome d'une pseudotuberculose du foie. Les cultures de cet organe ont révélé le *Corynebacterium pseudotuberculosis murium* (KUTSCHER), qui, pour différents motifs, a été considéré comme l'agent de la maladie. Ce micro-organisme n'a provoqué les pneumonies à foyers, régulièrement décrites par la bibliographie, que lorsqu'il a été administré oralement avec les aliments.

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(From the Laboratory of the Waterworks, Rotterdam).

AN IMPROVEMENT IN THE BACTO-TRYPTONE, SODIUM FORMATE MEDIUM FOR THE DETECTION OF *B. COLI*

by

T. FOLPMERS

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An improvement of the bacto-tryptone, sodium formate medium [1]), which has now been put on trial for nearly a year in the Laboratory of the Waterworks at Rotterdam, consists in lowering the content of crystal violet to half of the original amount and in raising the pH to 6.8.

The medium is now prepared as follows: 0.3 % bacto-tryptone, 0.04 % K_2HPO_4, 0.1 % HCOONa, 0.00005 % crystal violet, pH = 6.8. Dissolve in 1800 ml distilled water 30 grams bacto-tryptone, 4 grams K_2HPO_4 and 1.3 grams NaCl. Filter through Swedish filterpaper, add ± 13 ml n HCl. Sterilise in Koch's steriliser for an hour. Add 50 ml sterilised 20 % sodium formate solution, 5 ml sterilised aqueous crystal violet solution (1 : 1000) and sterile distilled water up to 2000 ml.

Cultivate in completely filled stoppered glass bottles at 44—45° C. in a 1 : 5 dilution of the medium. For this purpose supply the glass bottle with one fifth of its content with the culture medium, add the water to be tested either undiluted or in known dilution and fill in the latter case the bottle with sterile water. Test for indol and gas production after 24 and 48 hours. Gas production is often very slight; in such case attention has to be paid to an eventual growth of coli bacteria at the bottom of the glass bottle, which may be rendered visible by shaking the bottle gently.

Indol reagents: 2 grams p. dimethylamino-benzaldehyde in 190 ml 90 % alcohol and 40 ml 25 % HCl. Bring the reagent by means of a pipette as a thin layer over an aliquot of the culture solution in a test tube and heat this in the small flame of a Bunsen burner.

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1) cf. T. Folpmers, Antonie van Leeuwenhoek **6**, 22, 1939—1940.

(From the Department for Bacteriology and Experimental Pathology of the Institute of Preventive Medicine at Leiden).

A FILTERABLE VIRUS AS A CAUSATIVE AGENT OF EPIDEMIC HEPATITIS

by

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(Received January 14, 1944).

1. INTRODUCTION.

Many cases of so-called catarrhal jaundice are based on diffuse hepatitis. This disease, which can spread epidemically and especially in war time pandemically, is indicated by the name of epidemic hepatitis. Though its cause is not known, it is surmised that the agent is a filterable virus. Several arguments support this conception. The sometimes epidemical spreading, especially in winter, points to the infective nature of the affection. Nobody succeeded however, in pointing out visible micro-organisms as a cause, nor did the serological investigations with respect to the known pathogenic ones, among which we especially think of leptospirae, give any indications as to the presumable etiology. Attempts to transmit the disease on animals by inoculation with blood, urine or duodenal fluid failed with hardly any exception. Of late years however, some facts have been observed that might point to a virus etiology. For epidemic hepatitis has indeed been diagnosed after the injection of sterile human serum (FINDLAY, MC. CALLUM and MURGATROYD (5)), and after blood transfusion (JUNET, l.c. ZIEGLER (10)). After the inoculation of duodenal fluid of sufferers from epidemic hepatitis on the chorioallantois of the chick embryo, SIEDE and MEDING (7) and SIEDE and LUZ (6) made evident the existence of a filterable agent, which they could grow on in eight egg passages and which regularly caused the death of the embryos after five days.

VOEGT (9) tried to infect some persons by making them drink the duodenal fluid of sufferers from epidemic hepatitis or by subcutaneous injections of blood or urine. He did not succeed however, in exciting the typical clinical picture of the disease; particularly jaundice was always absent. He communicated, that after about four weeks a slight damage of the liver occurred, which was stated by burdening experiments and by chemical analyses of blood and urine.

Interesting but not yet proved experiments have been made by ANDERSEN and TULINIUS (1,2 ,3). They start from the supposition

that the enzootic hepatitis of pigs, which is of frequent occurrence in Denmark, has perhaps a causal connection with epidemic hepatitis of man. They base this hypothesis on the conformity of the histopathological changes in the livers in both diseases and on the fact that the epidemics of hepatitis in man occur along with those in pigs, which parellelism they prove by means of diagrams. At Copenhagen e.g. where the meat inspection is very severe, all icteric pigs are declared unfit for human consumption. In the country and in most other towns this is not the case and it is true that there epidemic hepatitis is much more frequent than at Copenhagen. In the case of one epidemic, they suggest to have indicated a butcher's shop, where the meat of an icteric pig had been sold, as a source of infection.

They tried to prove their hypothesis experimentally and suggest to have succeeded in transmitting the hepatitis of pigs on young and underfed pigs by feeding them with liver. Jaundice would appear already three days after the inoculation. Infection would also be possible by drinking duodenal fluid of diseased pigs. By oral inoculation they could transmit the hepatitis of pigs in four and the hepatitis of man in two passages. In both cases the histological changes of the liver agreed with those of the spontaneous cases in man and pigs. The bacteriological investigations always having a negative result, they suggest to have proved, that the epidemic hepatitis of man and pigs is caused by the same virus.

In this war, epidemic hepatitis appears again pandemically and in the winter of 1942/1943 we set up an investigation into the etiology of the disease. The result of this was, that by means of experiments on guinea pigs a filterable virus could be shown as a cause, which has already been briefly communicated elsewhere (Verlinde and Boer (8)). Without going further into the clinical picture of the patients, we shall dwell a little longer on the experimental investigations, which have again been extended since the preceding publication.

2. Inoculation experiments in guinea pigs.

So far inoculation experiments have been made with the material of eight patients suffering from epidemic hepatitis. In four of them we succeeded in demonstrating the virus in the blood during the initial fever as well as in the urine during the jaundice (see table 1).

As a rule we used the intraperitoneal, in guinea pig 160 and 162 the intracardial inoculation. The temperature was taken twice a day, *viz.*, at 9 o'clock a.m. and at 4 o'clock p.m. The guinea pigs that reacted positively upon the injection, showed a rise of temperature which was not very conspicuous, except the animals injected with the urine of patient 5. Sometimes only the afternoon temperature gave a distinct top. Once or twice a second top was observed some days afterwards (fig. 1).

Table 1.

Patient number	Material	Stage of the disease	Inoculated guinea pig	Reaction	Incubation period
5	urine (unfiltered)	jaundice 4 days	159	+	8 days
	,,	,,	160	+	8 ,,
	urine (Seitz filtrate)	,,	161	+	8 ,,
	,,	,,	162	+	8 ,,
6	blood (defibrinated)	fever	190	±	10 days
	,,	,,	191	±	
7	urine (unfiltered)	jaundice 1 day	217	—	
	,,	,,	218	—	
	urine (filtered)	,,	219	—	
	,,	,,	220	—	
10	blood (defibrinated)	fever	273	+	7 days
	,,	,,	274	+	7 ,,
	urine (unfiltered)	jaundice 2 days	309	—	
	,,	,,	310	+	6 ,,
12	stools (Seitz filtrate)	jaundice	353	—	
	,,	,,	354	—	
14	liver punctation (post	liver atrophy	287	—	
	,, (mortem)	,,	288	—	
15	urine (unfiltered)	jaundice	361	—	
	,,	,,	362	—	
16	urine (unfiltered)	jaundice 2 days	423	—	
	,,	,,	424	+	10 days
	throat-swill (Seitz	,,	425	—	
	,, filtrate)	,,	426	—	

On judging the temperature the course of the normal curve must be taken in consideration for each animal individually, so that it is advisable to registrate the temperature already some time before the injection. The normal temperature is not the same in every

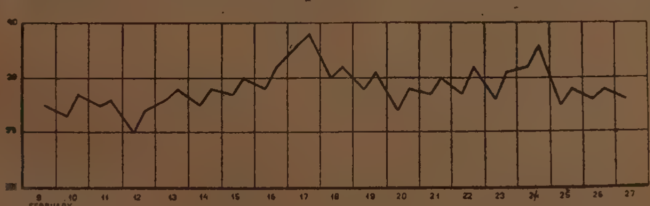

Fig. 1. February 9. intraperitoneal inoculation urine patient 5 (Seitz filtrate).

guinea pig and also in one single animal it may vary. On the day after the injection a certain rise of temperature is sometimes perceptible, which must be understood however, as a non-specific reaction to the injection. Generally this rise does not last longer than one day. For each inoculation at least two guinea pigs have

been used. One of them was killed when the temperature had risen considerably, in the other we watched the further course, and afterwards the animal could be used for immunity experiments. In the killed guinea pig, the organs were bacteriologically investigated, after which they could be used for inoculation experiments, when the result had been negative.

In order to be able to exclude in the blood and in the urine of normal persons a possible presence of an agent that might cause the same reaction in guinea pigs, we gave eight guinea pigs an intraperitoneal injection with 5 ml of the blood and the urine of four normal persons. This did not cause rises of temperature and inoculation experiments with the blood and the organs of three of them, which had been killed after 6, 8 and 10 days, had a negative course.

When the result of the bacteriological investigations of the killed guinea pigs was negative, the inoculation experiments were carried out on fresh guinea pigs. In this way, starting from patients numbers 5, 6, 10 and 16, respectively 11, 8, 6 and 7 passages were made. In the passage animals fever as a rule is evident; the temperature curve often shows two tops, sometimes even a period of fever, which may last mostly 3—5 and once 12 days (fig. 2 and 3).

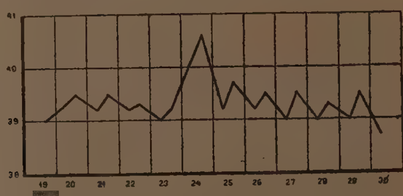

Fig. 2. Guinea pig 232: March 19. intraperitoneal inoculation blood plasma of guinea pig 209.

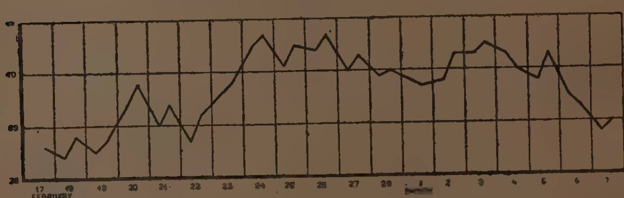

Fig. 3. Guinea pig 166: February 17. intraperitoneal inoculation liver guinea pig 159.

It may occur, as was the case in patients 6 and 10, that the guinea pigs, after the injection of urine or blood of a patient, do not react distinctly by a rise of temperature, or do not react at all. When the animals however, are killed 8—10 days after the injection, fever does occur in the second passage. Therefore it is possible that not all guinea pigs giving a negative reaction, are actually negative, but

that they might become positive as yet by one or more blind passages. Also in series inoculations it occurs repeatedly that only one of the two inoculated animals reacts with fever, whilst the next passage of such a clinically negative animal may be distinctly positive. So in fact the guinea pig is sensitive to the virus of epidemic hepatitis, but not in a high degree. We found young guinea pigs of a weight of 250—350 g the best suited for experimental researches with the virus.

The inoculation experiments have been made with blood, organs and bile during the fever period and with urine after this period. The blood has been taken by puncture of the heart; the organ suspensions have been prepared by grinding the organ with sterile sand and suspending it in 9 parts of a 0,9 % saline solution. The Seitz EK filtrate has been made of organ suspensions prepared in the way described above. The quantity of blood or of organ suspensions (unfiltered) injected, amounts to 1—5 ml, whilst we generally injected 5 ml of the Seitz filtrate. It appears from table 2, that the virus could be ascertained in the blood and in the organs during the fever period. After this the virus is apparently excreted with the urine during merely a short period, for in the guinea pig it could still be shown in the urine 5 days after the fever period, a fortnight after this period no more.

Table 2.

Material	number of guinea pigs	
	infected	positive
fresh blood	5	3
oxalated blood	5	3
red blood corpuscules	2	1
bloodplasm	2	2
liver (unfiltered)	50	32
liver (Seitz filtrate)	3	2
liver (dried)	2	2
kidney	2	1
spleen	2	2
brain	2	1
lung	1	1
bile	2	0
urine (5 days after fever)	1	1
urine (14 days after fever)	3	0

In patient 10 we did not succeed in demonstrating the virus in the urine a week after the cure of jaundice. In patient 16, on the other hand, the virus was still present in the urine a week after this cure.

The method of inoculation generally followed, is the intraperitoneal one, but it is also possible, though less constant in result, to infect guinea pigs by the intracardial, subcutaneous, oral and intracerebral route. The average incubation period after the in-

traperitoneal, intracardial and subcutaneous inoculation amounts to 6—8 days. The shortest interval observed is 3 days. After the oral inoculation (the animals being fed with virulent liver of guinea pigs), it amounted to 13 days. Twice we tried to effect an infection by contact by putting a guinea pig in a hutch together with a guinea pig with fever. One of these animals reacted with fever after 15 days. The length of this period agrees with the incubation period given for man (2—4 weeks).

3. Post mortem examination in the guinea pig.

The disease in the guinea pig seldom has a fatal course. In the few guinea pigs that died and in those, killed at different moments after the inoculation, we mostly did not find macroscopical changes. During the fever period the spleen may be swollen. Then the liver too is sometimes swollen and its surface may have a light yellow spotted aspect. In some cases after the fever period gray or white-yellow to ochreous, irregular foci are found, which on histological examination correspond with foci of atrophy and necrobiosis. Once we found a diffuse atrophy of a whole liver lobe, which had a yellow aspect. The focal changes are intralobular, may expand over the whole lobulus and sometimes fuse with the neighbouring lobuli, showing the same alterations. The process begins with swelling and dissociation of liver cells, and vacuolisation of the protoplasm. The taking of the stain by the protoplasm grows less and may disappear nearly completely, so that the focus assumes a transparent aspect.

Subsequently a diminuation of the liver cells may occur. The nuclei mostly remain unimpaired, but sometimes symptoms of degeneration occur. In or around the foci, sometimes through the whole liver, a fatty infiltration can be found. The cellular reaction is not conspicuous and consists of a slight perilobular infiltration with lymphocytes, which sometimes may be found on the edge of the foci. In the foci, the number of Kupffer cells may be increased. In the spleen, many megacaryocytes and plasmacells may be present, the reticulum often being loaded with little drops of fat. In the kidneys slight degenerative changes of the epithelium of the tubes are found.

We want to point to the fact that some of these changes may be absent. It may even occur that the pathological findings are perfectly negative. On the other hand we sometimes saw the changes in the liver described in guinea pigs that had not reacted to the inoculation with fever. The changes found by us in the liver of the guinea pig agree with those described in man.

4. Inoculation experiments in mice and rats.

When a guinea pig virus is transmitted to mice and rats either by the intraperitoneal, subcutaneous, intracerebral or oral route, no clinically perceptible morbid symptoms of fever appear, nor do we see typical alterations on autopsy. The virus however, may remain active when inoculated in series once a week. Usually the liver is

also used for the inoculations. As in these animals no criteria are observed that would prove the presence of the virus, it is only possible to demonstrate the virus by the inoculation of guinea pigs. Attempts to obtain a neurotropic virus by intracerebral route, such as is proceeded for the neurotropic virus of yellow fever, have not had any result so far. It has been possible to grow the virus on mice and rats during 5 passages, in the passage following this could not be demonstrated any more. Starting however from the virus of egg membrane eight cerebral mouse passages could be made so far.

5. Properties of the virus.

In 50 % glycerol the virulence can be retained at a temperature of 0—4° C. for 1—3 months. One strain was still virulent after 83 days, another having lost its virulence already after 51 days.

Dried virus, which is obtained by grinding the fresh liver of a guinea pig killed during the fever period and drying it in a vacuum exsiccator with sulphuric acid and anhydrous phosphoric acid at 4° C., proved to be still virulent after having been kept in a refrigerator for 33 days; after 63 days it had lost its virulence. When however, the virus is kept at —16° C., the virulence can be retained for at least half a year.

With a virulent suspension of liver, which has been heated at 55° C. for half an hour, it is not possible to excite a reaction in guinea pigs. So the virus soon looses its virulence by heat.

After the action of 0.1 % formaline at 37° C. a liver suspension was still able to excite fever in the guinea pig. This was not possible in a growth on egg membrane. After the liver suspension had been kept at 4° C. for another week, the virus proved to be inactivated.

The virus can easily be filtrated through a Seitz EK filter. Already in the first experiments we succeeded in exciting fever with filtrated urine, whilst afterwards it has also appeared that filtrated suspensions of liver and of egg membrane could excite this reaction as well. It is true, that the filtration is attended with a loss of virulence, which a longer incubation period makes apparent as well as with a shorter fever period after the inoculation with filtrated material in comparison with the same, but unfiltrated material.

Generally the virulence to the guinea pig is not high; 57 % out of about 100 passage guinea pigs reacted with distinct fever, 18 % reacted doubtfully and 25 % did not react at all.

We believe to have traced in our experiments a certain influence of the season on the sensitiveness of the guinea pig. In the winter months the inoculations give a more conspicuous reaction, while in summer they become effective with the greatest difficulty and only a strain of strongly virulent material can maintain itself in summer, whilst the less virulent ones are lost. These observations agree with the epidemiology in man; epidemic hepatitis occurs especially in winter.

6. The growth of the virus on the egg membrane.

We have tried to grow the virus on the chorioallantois of the chick embryo, according to the method of Burnet (4). After an incubation time of 11 days, the eggs are inoculated, for which we used an artificial air space; the final inoculation taking place after three more days. We started from a virulent liver of a guinea pig out of the series of patient number 5. About 20 egg passages have been made and the virus can still be demonstrated in the egg membrane and, though less apparent, in the liver and the brain of the embryo, by inoculation of the guinea pig (table 3).

Table 3.

Egg passage		Guinea pig	Reaction	Incubation period
Passage-number	Material			
4	embryo liver	292	+	5 days
	"	304	+	7 "
	allantois	313	—	
	"	335	+	8 "
	"	336	—	
7	embryo liver	347	+	4 "
	"	348	—	
	allantois	349	+	12 "
	"	350	+	8 "
11	"	371	—	
	"	372	—	
	"	373	+	4 "
14	embryo liver	405	+	10 "
	"	406	—	
	embryo brain	407	—	
	"	408	+	7 "
16	allantois	415	+	5 "
	"	416	+	8 "
	"	417	+	5 "
	"	418	+	5 "
20	"	537	+	5 "
	"	538	+	7 "

Also the direct inoculation of eggs with filtrated urine (patient 16) has now been succesful.

Contrary to the results of Siede c.s. (6, 7) our infected embryos as a rule do not die, at least not within 5 days after the inoculation.

Apparent changes, besides hyperaemia, sometimes local edema and small hemorrhages have not been found so far on the egg membranes. In the smears of the allantois, which have been stained by the Gram or Giemsa-method, we sometimes found in and around the cells large quantities of small round strongly coloured bodies, which have not been found in normal preparations. It is not improbable that these are elementary bodies of the virus.

A virulence experiment has been carried out with the 16th allantois-passage. The allantois was ground and diluted with a 0,9% saline-solution till 1/10, 1/100, 1/1000, 1/10.000 and 1/100.000. Of each dilution 5 ml has been intraperitoneally injected into two guinea pigs. The dilution of 1/1000 still caused some rise of temperature. This was not the case with higher dilutions.

7. IMMUNITY.

Guinea pigs that are infected with virulent material for the second time, do not react unless they are entirely recovered from the first infection. The moment in which the state of immunity sets in seems not only to depend on the date of the first infection, but also on the length of the fever period (Table 4).

Table 4.

Guinea pig.	Time between the first infection and the reinfection	Time between the last day of fever and the reinfection	Immunity
161	20 days	5 days	—
162	20 ,,	12 ,,	+
171	34 ,,	25 ,,	+
178	26 ,,	no fever	+
187	22 ,,	15 days	+
191	21 ,,	14 ,,	+
198	19 ,,	12 ,,	—
216	31 ,,	26 ,,	+
222	25 ,,	no fever	+

All guinea pigs mentioned in Table 4 have been reinfected with the same infectious material (liver of guinea pigs), which in normal guinea pigs excited a clear-cut fever reaction.

The first infections of guinea pigs 161 and 162 took place simultaneously. Both are reinfected 20 days later, but only guinea pig 162 is immune. The difference between the two animals consists in number 161 having had a long fever period and number 162 a short one, so that the reinfection of number 161 took place already 5 days after the last day of fever, whilst in number 162, 12 days passed between the last day of fever and the reinfection. Guinea pig 198, in the case of which also 12 days passed between the last day of fever and the reinfection, is not yet immune. So the shortest lapse of time after which a distinct immunity can be shown, must be rated at 12 days after the period of fever. The other guinea pigs have not reacted to the reinfection and so they are probably immune.

We cannot be certain whether this is really the case for we have noted that in normal guinea pigs the infection becomes effective only for 57 % as well. The fact, however, that a number of these animals did react with fever to the first inoculation, prove that they have been sensitive to the virus. Their not being sensitive to the

second infection points to an immunity obtained as a result of the first infection.

When the first infection has not excited fever, as in numbers 178 and 222, even then immunity may appear. This can also be explained by the animals being already naturally immune to the virus. So it is difficult to make out here whether the immunity is a result of the first infection. Yet this is probable, at least in one guinea pig, as neutralizing antibodies have been shown in the blood serum and these do not occur in the normal guinea pig.

For the detecting of neutralizing antibodies three or more suspensions of a virulent liver of a guinea pig are made, e.g. in the dilution 1/10, 1/20 and 1/50. To 1 ml of the virus dilution 1 ml of undiluted serum that is to be investigated, is added. The mixtures are kept at 37° C. for 2 hours and at 4° C. for half an hour; after that they are intraperitoneally injected into guinea pigs. In order to control the activity of the virus a same number of virus dilutions is mixed with a dilution of 0,9 % saline solution, which mixtures are treated in the same way. The temperature of the guinea pigs was taken twice a day during a fortnight. When the guinea pigs which have been injected with the virus serum mixtures react with fever, we can conclude that there are no antibodies. If the controls do react and these animals not, it is very probable that the serum contains antibodies.

However, as long as we do not succeed in obtaining a virus-fixe which excites fever in every case, the neutralization experiment cannot be considered as sufficiently reliable. We had hoped to find the mouse an animal suited to the neutralization experiment, but in this animal no typical reaction occurs from which the result of the experiment could be concluded. The results of the neutralization experiments with sera of patients and guinea pigs has been summarized in table 5. The virus dilutions used were 1/10, 1/20 and 1/50. With each mixture two guinea pigs were injected, so for every serum 12 guinea pigs have been used (6 for the virus-serum mixtures, 6 for the control mixtures).

It appears from table 5 that the neutralizing antibodies can be traced from the 13th day of the fever-period, *i.e.*, nearly simultaneously with the appearance of the resistence to the reinfection.

Guinea pigs that have not reacted with fever may yet possess antibodies. Some sera of patients showed a weak complement-fixation-reaction with an antigen prepared by keeping a 10 % suspension of a fresh virulent liver of a guinea pig at 37° C. for 24 hours and at 4° C. for two more days, and subsequently centrifugating it at 3000 r.p.m. for 15 minutes.

The technics used for the reaction were those of Besredka. The results are expressed in the number of unities of complement. One unity of complement is 0.1 ml of fresh guinea pig serum 1/15 which is fixed by 1 ml of the patient-serum. Patients-sera 1, 6, 7, 8, and 14 fixed respectively 10, 15, 15, 20 and 10 unities, 5 normal sera fixed 0 unities.

Table 5.

Serum		Number of days after the period of lever	Result of the experiment
Patient	1	90	+++
„	5	24	++
„	6	18	++
„	7	17	+
„	8	36	++
„	9	25	+++
„	14	unknown	++
Guinea pig	160	5	—
„	166	36	++
„	177	no reaction	+
„	179	16	+
„	193	13	+
„	208	26	+++
„	215	no reaction	—
„	221	„	++
„	222	„	+
„	223	„	+
„	226	21	++
„	227	15	++
„	229	22	+
„	233	14	++
„	243	14	+

— all mixtures positive (no neutralization).
\+ mixtures 1/10 and 1/20 positive (weak neutralization).
++ mixtures 1/10 positive (moderate neutralization).
+++ all mixtures negative (strong neutralization).

Summary.

In four patients suffering from epidemic hepatitis we succeeded in isolating from the blood during the fever period and from the urine during the jaundice, a filterable virus, which is pathogenic to the guinea pig and which can be inoculated to this animal in different ways, but by preference intraperitoneally. Fever, which sometimes lasts only one day, is the only morbid symptom observed in guinea pigs.

In these animals the virus can be shown in the organs and in the blood during the fever period; after that it is excreted with the urine during apparently a short period. The virus has been grown on the chorioallantois of the chick embryo so far during 20 passages. The virus is resistant to glycerol, drying and low temperatures, not to formaline and heating.

In the livers of the guinea pigs focal changes (degeneration, dissociation, necrobiosis, yellow liver-atrophy) may be found. After dulling through the disease immunity occurs, whilst in the serum of recovered patients and guinea pigs neutralizing antibodies can be detected.

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(Aus dem Centraalbureau voor Schimmelcultures, Baarn).

BESCHREIBUNG EINIGER NEUER PILZARTEN AUS DEM CENTRAALBUREAU VOOR SCHIMMELCULTURES, BAARN (NEDERLAND)

VIII. MITTEILUNG

von

F. H. VAN BEYMA THOE KINGMA

(Eingegangen am 7 Februar, 1944).

Chaetomium minimum nov. spec.

Dieser Pilz wurde dem C.B.S. von Herrn Ir. E. HARMSEN (Kampen) zugeschickt, der denselben aus sauren Podsol-Böden wiederholt isolierte. Vor allen Dingen BAINIER (2) und später CHIVERS (6) haben sich mit dem Studium der Gattung *Chaetomium* befasst und zahlreiche Arten unterschieden und beschrieben. Unser Pilz konnte jedoch mit keiner der dort aufgezählten oder in der Sammlung des C.B.S. vorhandenen Arten identifiziert werden. Wegen der geringen Grösse der Perithezien haben wir ihn *Ch. minimum* genannt.

Die Perithezien sind ziemlich klein, kleiner wie es bei den meisten Arten dieser Gattung gewöhnlich der Fall ist. Die grösste Aehnlichkeit zeigt unser Pilz noch mit *Ch. aureum* Chivers; von diesem unterscheidet er sich jedoch durch die schwarze Farbe der reifen Perithezien und die grösseren, ausserdem abweichend gestalteten Sporen. Die Grösse der Askosporen beträgt bei *Ch. minimum* im Mittel 11,9 × 5,6 μ, bei *Ch. aureum* 9,8 × 5,4 μ. Bei letztgenannter Art sind sie mehr oder weniger oval, bei erstgenannter spindelförmig, oft mit einer geraden Seite. Sie treten aus den Perithezien in geraden oder gekrümmten, bis

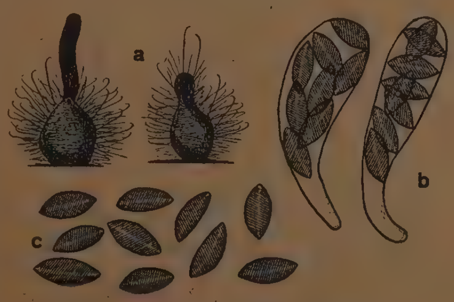

Fig. 1. *Chaetomium minimum*.
a. Perithezien. Vergr. 80 ×.
b. Asci. Vergr. 750 ×.
c. Askosporen. Vergr. 750 ×.

40 μ breiten Säulen hervor. Der Haarschopf der Perithezien fällt nicht besonders auf, er besteht meist aus einigen steifen, braunen, inkrustierten, 4 μ dicken Borsten, welche an der Spitze bischoffsstabähnlich umgebogen sind.

Auf Agar-Nährböden bildet der Pilz wollige bis strähnig-wollige, gelbbraune Decken, wobei ein in den Nährboden hinein diffundierender Farbstoff denselben dunkelbraun bis fast schwarzbraun färbt. Das beste Wachstum erzielt man auf Lupinenstengeln, diese sind innerhalb einer Woche von zahlreichen Perithezien überwachsen, ohne Luftmyzel.

In bestimmten Böden scheint *Ch. minimum* eine wichtige Rolle zu spielen. In einem Schreiben an das C.B.S. teilt Ir. E. HARMSEN mit, dass dieses *Chaetomium* der wichtigste Zellulose-Zersetzer in sauren Podsol-Böden während der ersten Phase des Abbruchs von frisch untergepflügtem, zellulosereichem Material (Stroh, Spreu, Papier, Abfälle, u.s.w.) in den Niederlanden sei; sie enthalten bisweilen bis zu 20.000 Keime/Gramm.

Die Beschreibung lautet wie folgt:

Chaetomium minimum nov. spec.

Perithezien braunschwarz bis schwarz, (120—130) × (70—100) μ, ellipsoidisch bis gedrückt-kegelförmig. Haarschopf aus verhältnismässig spärlichen, septierten, dunkelbraunen, steifen, inkrustierten, 2—3 μ dicken Borsten bestehend, welche an der Spitze bischoffsstabähnlich umgebogen sind. Rhizoiden spärlich entwickelt, seitliche Haare einfach, pfriemenförmig, septiert, braun, inkrustiert, 4 μ dick.

Asci keulenförmig, kurz gestielt, vergänglich, 36—45 μ lang (pars sporif. 33 μ) und 10—12 μ breit.

Sporen spindelförmig, oft mit einer geraden Seite, olivenbraun, (10,7—13,3) × (5—6) μ — meist (12—13) × (5—5,7) μ — in langen, schwarzen, bis 40 μ dicken, geraden oder gekrümmten Säulen austretend.

Hab. Aus sauren Podsol-Böden aus der Gegend von Kampen (Niederl.) (Ir. E. HARMSEN).

Lateinische Beschreibung.

Peritheziis atrobrunneis vel atris, minutis, (120—130) × (70—100) μ, ellipsoideis vel obtusiter coniformibus. Setis raris, rigentibus et atrobrunneis, in acumine curvis. Pilis rhizoideis modicis. Filis lateralibus subuliformibus, brunneis. Ascis fustiformibus, in brevibus pediculis sedentibus, fragilibus, 36—45 μ longis (pars sporif. 33 μ), 10—12 μ latis. Sporulis fusiformibus, saepe inaequilateralibus, colore brunneo oleagino, (10,7—3,3) × (5—6) μ, — plerumque (12—13) × (5—5,7) μ —, se exserentibus stylis longis, usque ad 40 μ latis, distichis.

Piptocephalis macrospora nov. spec.

Aus dem Laboratorium der ,,Specerijen-Malerij De Kö-ver'' in Boxmeer (Niederl.) erhielt das C. B. S. verschimmelten Nelkenersatz

zur Untersuchung. Aus dem mit Wasser zu einer feuchten Masse verrührten Pulver, das auf feuchtem Filtrierpapier in einer Petrischale einige Tage bei 24° C. gehalten wurde, entwickelte sich ein Pilz, der alsbald als zur Gattung *Piptocephalis* gehörig erkannt werden konnte. Da *Piptocephalis* bekanntlich auf anderen Mucorineen schmarotzt, konnte die Anwesenheit von Pilzen dieser Familie vermutet werden. Bei der Abimpfung wurden in der Tat zwei Mucorineen in den Kulturgefässen festgestellt und zwar *Mucor racemosus* Fres. und *Absidia ramosa* (Lindt) Lendner. Als einer der am meisten geeigneten Nährböden erwies sich sterilisierte Möhre. Die Herstellung von Reinkulturen gelang leicht, da die Sporangienträger von *Piptocephalis* sich ziemlich hoch über die Pulvermasse erheben, sodass mit Hilfe einer Pinzette die Spitzen der Träger leicht erfasst und auf Möhre übertragen werden konnten. Die Fruchthyphen des Pilzes bilden weder Rhizoiden noch Ausläufer; sie entspringen unmittelbar aus dem Myzel. Die Verzweigung der Sporangienträger ist die für *Piptocephalis* typische, nämlich wiederholt dichotom. Die septierten Gabeläste sind anfangs hyalin, werden aber bald braun und weisen eine feine Längsstreifung auf. Die letzten Verzweigungen enden in Zellen, die wir nach ZYCHA Basalzellen nennen wollen. Diese Basalzellen sind kugelig, 7—8 μ im Durchmesser und ringsum mit zahlreichen Höckerchen, woran die Teilsporangien sitzen, versehen. Sie fallen leicht ab, sodass man sie überall im mikroskopischen Präparat umherschwimmen sieht.

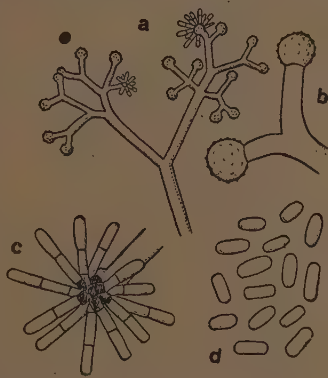

Fig. 2. *Piptocephalis macrospora.*
a. Sporangienträger. Vergr. 245 ×.
b. Basalzellen. Vergr. 750 ×.
c. Köpfchen. Vergr. 750 ×.
d. Sporen. Vergr. 750 ×.

Was die systematische Stellung anbetrifft steht unser Pilz zweifellos der *Piptocephalis cylindrospora* nahe. Der Unterschied besteht hauptsächlich hierin, dass bei unserem Pilze die Grösse der Basalzellen 7—8 μ, die der Sporen 7 × (3—3,3) μ beträgt, gegen 3—4 μ für die Basalzellen und 4 × 2 μ für die Sporen bei *Piptocephalis cylindrospora*. Auf Grund dieser grösseren Abmessungen haben wir den Pilz *Piptocephalis macrospora* genannt (9, 11).

Die Beschreibung des Pilzes gestaltet sich folgendermaassen:

Piptocephalis macrospora nov. spec.

Myzel ohne Ausläufer.
Sporangienträger aufrecht, mehrfach dichotom verzweigt, mit rechtwinklig abzweigenden, nach oben immer kürzer werdenden Gabel-

ästen, anfangs farblos, später braungelb mit undeutlich gestreifter Membran, septiert, mit farblosem Inhalt.

Basalzellen kugelig, 7—8 μ im Durchmesser, allseitig mit kleinen Höckerchen versehen, leicht, jedoch nicht immer, abfallend.

Teilsporangien zahlreich, etwa 15—20 μ lang, 2—3 gliedrig, gerade, aufrecht.

Sporen zylindrisch, hyalin, glatt, (6,3—8,7) × (3—3,3) μ, meist 7 × (3—3,3) μ.

Zygosporen unbekannt.

Hab. Parasitisch auf *Mucor racemosus* Fres. und *Absidia ramosa* (Lindt) Lendner, wachsend auf Nelkenersatz (Specerijen-Malerij De Körver, Boxmeer, Niederl.).

Lateinische Beschreibung.

Mycelio sine ramis. Conidiophoris erectis, saepe dichotomis, primo sine colore, deinde brunneoflavis, membrano obscuriter striato praeditis. Cellulis basalibus globosis, 7—8 μ crassis. Sporulis cylindriformibus, hyalinis, levibus, (6,3—8,7) × (3—3,3) μ; plerumque 7 × (3—3,3) μ. Zygosporula ignota sunt.

Scopularia corsicana nov. spec.

Von Frl. Dr. H. C. Koning (Baarn) erhielt das C. B. S. einen Pilz, isoliert aus dem Holze von *Pinus nigra corsicana*. Es handelte sich um etwa dreijährige Kiefer mit wahrscheinlich durch Frost beschädigten Trieben, deren Nadeln an den Spitzen braun verfärbt waren. Im Verlauf desselben Jahres hatten die Bäumchen sich jedoch völlig erholt.

Der betreffende Pilz wurde leicht als zur Gattung *Scopularia* Preuss em. G. Goidànich (Syn. *Leptographium* Lagerberg et Melin) gehörig erkannt. Die Merkmale: Konidienträger aus einem einzelnen Faden aufgebaut mit dunkelbraunem Stiele, der in einen penicilliumartig verzweigten Pinsel übergeht und die zu grossen Tropfen zusammenfliessenden Konidien waren sämtlich anwesend. G. Goidànich (8) hat die Gattung *Grosmannia* gegründet als perfekte Form von *Scopularia*. Eine Askus-Form ist jedoch bis jetzt in unseren Kulturen nicht aufgetreten. Der Pilz konnte mit keiner der beschriebenen oder in der Sammlung des C.B.S.

Fig. 3. *Scopularia corsicana*.
a. Konidienträger. Vergr. 245 ×.
b. Endästchen der Konidienträger. 750 ×.
c. Konidien. Vergr. 750 ×.

vorhandenen *Scopularia*-Arten identifiziert werden. Von den ihm am nächsten stehenden Arten: *Sc. tenuissima* (Cda) G. Goid., *Sc. penicillioides* (Grosm.) G. Goid., *Sc. Lundbergii* (Lag. et Melin) G. Goid., oder *Sc. pini* G. Goid. weicht er entschieden ab. *Sc. tenuissima* besitzt spindelförmige Konidien, *Sc. penicillioides* gekrümmte Konidien, *Sc. Lundbergii* weist grössere Konidien auf und *Sc. pini* endlich hat anders gestaltete Konidienträger.

Er wurde deshalb als neue Art beschrieben. Die Beschreibung lautet:

Scopularia corsicana nov. spec.

Hyphen von kräftigem Wuchs, 2,7—3 μ breit, septiert, leichtbraun gefärbt, im Alter nachdunkelnd bis fast schwarz.
Konidienträger 4—6 μ breit, mit braun gefärbtem Stiel, aufrecht, septiert, nur am Scheitel mehrfach verästelt. Endästchen 10—16 μ lang und 2 μ breit, hyalin bis leicht gefärbt, zahlreich, dicht gedrängt stehend und die Konidien in grossen Massen abschnürend.
Konidien massenhaft, in feuchter Umgebung zu grossen schleimigen Massen zusammenfliessend, einzellig, ellipsoidisch, von verschiedener Grösse, (4—12) × (2—4,7) μ, meist (5,3—6,3) × (2,7—3,3) μ, ohne Oeltröpfchen, jedoch mit einer Vakuole oder mit undeutlichem Inhalt.
Hab. Aus dem Holze von *Pinus nigra corsicana* (Frl. Dr. H. Koning, Baarn).

Reinkulturen:

Auf Bierwürze-Agar in einer Petrischale nach 6 Tagen: schnellwüchsig, Schale ganz bewachsen, vom Impfstück aus flach dem Agar anliegende schwarze Hyphenstränge. Ueberwuchs von kleinen, wolligen, hellbräunlichen Myzelflocken, welche die fertilen Hyphen enthalten. Farbe der Kultur gegen das Licht olivgrün. Geruch säuerlich. Unterseite olivgrün. Desgl. nach 14 Tagen: eine geschlossene schwarze Decke, welche überdeckt wird von einer flachwolligen, leichtbraunen Schicht, bestehend aus zahlreichen kleinen, an den Trägern sich entwickelnden Sporentröpfchen. Unterseite grauschwarz.

Auf Röhrchen nach 14 Tagen:

Auf Bierwürze-Agar: eine schwarze Decke mit flachwolligem Ueberwuchs von zahlreichen, Sporenköpfchen bildenden Konidienträgern.

Auf Kirsch-Agar: wie vorige.

Auf Möhre: das Stück ganz bewachsen von einer schwarzen, glatten, feuchten Haut, stellenweise mit Konidienträgern.

Auf Kartoffelstück: wie vorige.

Lateinische Beschreibung.

Conidiophoris 4—6 μ latis, pediculo brunneo praeditis. Acumine ramatis sicut penicillium. Ramulis extremis 10—16 μ longis, 2 μ latis. Conidiis

consistentibus una cella, ellipsoideis, variae crassitudinis, (4—12) × (2—4,7) μ — plerumque (5,3—6,3) × (2,7—3,3) μ —, eguttulatis, attamen vacuola praeditis vel ambigua protoplasma continentibus.

Gliocladium cibotii nox. spec.

Diesen Pilz erhielt das C. B. S. von Frl. M. F. Habekotté in Delft, wo er von den Haaren des Baumfarnes *Cibotium Schiedei* isoliert wurde. Bei üppigem Wachstum bildet er gern Hyphenbündel, von denen die Konidienträger allseitig abgehen. Diese Konidienträger können einfach sein, in den meisten Fällen jedoch sind sie gabelförmig verzweigt mit 2 oder 3 Aestchen. Dadurch nähert sich der Pilz der Gattung *Verticillium* und es fällt manchmal schwer, beim Studium der systematischen Stellung für dergleiche Organismen die richtige Wahl zu treffen. Es sei daher gestattet auf das Verhältnis zwischen *Gliocladium* und *Verticillium* an dieser Stelle etwas näher einzugehen.

In der ursprünglichen Diagnose von *Gliocladium* Corda entstehen die Konidien nicht in Ketten, sondern einzeln nacheinander, schliesslich durch Schleim zu einem Köpfchen verklebt, im Gegensatz zu *Penicillium*, wo die Sporen in Ketten erzeugt werden, welche nicht verschleimen. Später wurden auch Arten beschrieben, welche die Konidien in Ketten bildeten, die sich dann schliesslich ebenfalls zu verschleimten Köpfchen zusammenballten. Die Verzweigung der Konidienträger bei *Gliocladium* soll mehr oder weniger penicilliumartig sein, jedenfalls nicht in mehreren Wirteln übereinander wie bei *Verticillium*, sondern höchstens gabelförmig mit 2—3 Endästchen. Nun können auch Arten aus dem Formenkreise der Verticillien eine ähnliche einfache Verzweigung der Konidienträger aufweisen, welche auch auf den besten Nährböden nur selten darüber hinaus geht, sodass man im Zweifel sein kann, ob ein *Gliocladium* oder ein *Verticillium* vorliegt. In dem Falle kann dann die Anwesenheit von braunen Chlamydosporen entscheidend sein für die systematische Stellung eines solchen Pilzes. Sind diese nämlich im Myzel vorhanden, so fallen die betreffenden Organismen in den Formenbereich von *Verticillium dahliae* Klebahn. Von van Beyma (4) wurden dergleiche Formen beschrieben als *Verticillium dahliae* Klebahn forma *restrictum* und *Verticillium dahliae* Klebahn forma *zonatum*. Als Zwischenform zwischen *Verticillium* und *Gliocladium* sei *Gliocladium roseum* Bainier genannt. Foëx (7) fand die *Verticillium*-Form dieses Pilzes auf Kartoffeln; hier kann sie eine Fäule verursachen wobei im Gewebe zahlreiche Sklerotien entstehen. Nachdem Foëx den Pilz dem C. B. S. zugeschickt hatte, wurde er von van Beyma als ein richtiges *Verticillium* betrachtet und unter dem Namen *Verticillium Foëxii* nov. spec. beschrieben (3). Die *Penicillium*-Form war seinerzeit von Link *Penicillium roseum* genannt worden, wurde jedoch später von Bainier (1) auf Grund der in feuchter Umgebung sich bildenden Sporenköpfchen zur Gattung *Gliocladium* gebracht. Da beide Formen nebeneinander

vorkommen können, ist es schwer zu entscheiden, wohin der Pilz eigentlich gehört; der ältere Name *Gliocladium* mag daher vorläufig den Vorzug haben. Abbildungen beider Formen, der *Acrostalagmus-* (= *Verticillium*) Form und der *Penicillium-* Form findet man bei Thom in seiner Monographie über *Penicillium* (10).

Wenn wir nun bei unserem Pilze die hier erwähnten Erwägungen in Betracht ziehen, kann von einer eigentlichen *Verticillium*-Form wohl nicht die Rede sein, da die Zahl der Aestchen 3 nicht übersteigt, die Wirtel also möglichst einfach sind und mehrfach quirliche Verzweigung nicht auftritt. Auch fehlen schliesslich braune Chlamy-

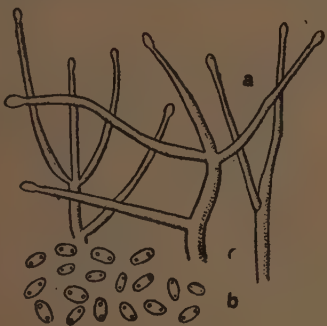

Fig. 4. *Gliocladium cibotii*.

a. **Konidienträger. Vergr. 750 ×.**
b. **Konidien. Vergr. 750 ×.**

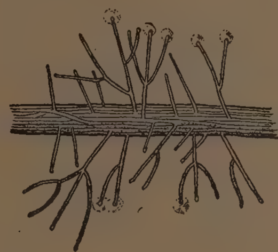

Fig. 5. *Gliocladium cibotii*.

Hyphenbündel mit Konidienträgern. Vergr. 245 ×.

dosporen. Daher kann kaum ein Zweifel darüber bestehen, dass der Pilz am besten zu *Gliocladium* gestellt wird.

Auf allen Nährböden wächst er anfangs mit einem weissen, etwas wolligen Myzel, das alsbald in den tieferen Schichten eine dunkelbraune bis schwarze Farbe annimmt. Die Konidienbildung war anfangs eine sehr üppige, die Sporenköpfchen flossen zu unzähligen Tröpfchen zusammen. Im Verlauf der Weiterzüchtung hat jedoch diese Konidienbildung etwas nachgelassen, sowie auch die anfängliche Neigung zur Erzeugung von aufstehenden Hyphenbündeln.

Die Beschreibung des Pilzes lautet wie folgt:

Gliocladium cibotii nov. spec.

Hyphen zart, 2—3 μ dick, leicht gefärbt, gern zu Hyphenbündeln zusammentretend, welche dann eine grünbraune bis braunschwarze Farbe aufweisen.

Konidienträger von den Hyphenbündeln in grosser Zahl abgehend, 80—100 μ lang und 2—3 μ dick, wenig verzweigt, meist gabelförmig mit 2—3 Sterigmen.

Sterigmen gerade, pfriemenförmig, 20—40 μ lang, an der Basis 2 μ breit, meist 2—3 an der Zahl, opponiert oder wirtelig stehend.

Konidien massenhaft, zu falschen Köpfchen verklebt, hyalin, einzellig, ellipsoidisch, (4—6) × (2—2,7) μ, meist (4—4,7) × (2,3—2,7) μ, mit zwei Oeltröpfchen.

Hab. Isoliert aus den Haaren des Baumfarnes *Cibotium Schiedei* (Frl. M. F. HABEKOTTÉ, Delft).

Reinkulturen:

Auf Bierwürze-Agar in einer Petrischale nach 1 Monat: Kolonie 7 cm im Durchmesser, im Zentrum mit mehreren, bis 1 cm hohen, weissen Hyphenbündeln. Um das Zentrum herum eine grauschwarze filzige Decke mit mehreren tiefen, radiären Falten; hier findet üppige Konidienbildung statt. Der Rand besteht aus einer breiten Zone von dünnen, farblosen, sterilen, bis 1 cm hohen Hyphenbündeln. Geruch schwach würzig. Unterseite im Zentrum schwarz, sonst blass gelblich.

Auf Röhrchen nach 14 Tagen:

Auf Bierwürze-Agar: unten im Röhrchen eine weisse, filzige Decke, ohne oder mit spärlicher Konidienentwicklung.

Auf Kirsch-Agar: die Agar-Oberfläche überdeckt von kleinen, zahlreichen, weisslichen Sporentröpfchen. Stellenweise erheben sich bis 3 mm hohe, in Büscheln beisammen stehende Hyphenbündel, welche zahlreiche Konidienträger erzeugen.

Auf Möhre: das Stück ganz bewachsen von einer filzigen, weissen Decke, von welcher zahlreiche Hyphenbündel sich erheben.

Auf Kartoffelstück: wie vorige.

Auf Kartoffel-Agar: das Wachstum stimmt mit dem auf Kirsch-Agar überein, üppige Konidienbildung.

Auf Haferflocken-Agar: wie vorige, nur bildet sich am Glase ein schwarzer Rand, der bei Kartoffel-Agar fehlt.

Lateinische Beschreibung.

Hyphis saepe conjunctis densis fascibus atrobrunneis. Conidiophoris permultis, ex hyphorum fascibus exeuntibus, 80—100 μ longis et 2—3 μ crassis, plerumque furcae in modo ramatis, 2—3 sterigmis praeditis, rectis, subuliformibus, 20—40 μ longis, basei 2 μ latis, oppositis vel vorticis in modum positis. Conidiis numerosissimis, in caespitulis cohaerentibus, hyalinis continuis ellipsoideis, (4—6) × (2—3,7) μ, plerumque (4—4,7) × (2,3—2,7) μ, cum duobus guttulis oleaginis.

Margarinomyces mutabilis nov. spec.

Das C. B. S. erhielt diesen Pilz unter No 1661 von Dr. H. WINDISCH (München) der ihn im Sommer 1942 aus einer zur biologischen Untersuchung eingesandten Flusswasserprobe isolierte.

Auf Agar-Nährböden wächst er mit zottigem Myzel, bestehend aus Hyphenbündeln, welche mit ihren zahlreichen Konidienträgern die Konidien in grossen Mengen abschnüren. Dagegen ist das Wachstum auf Kartoffelstück und Möhre schlecht; hier entstehen nur glatte, submerse Häute ohne Konidienentwicklung. Anfänglich ist

die Pilzdecke auf Bierwürze-Agar weiss, wird dann innerhalb 4 Wochen durch massenhafte Bildung, vom Zentrum aus, von endständigen und gewöhnlichen Konidien schwarz, mit einem Ueberwuchs von weissem Myzel mit kurzen, verfilzten Hyphenbündeln. Die endständigen Konidien sind meist einzellig. Sie entstehen am Ende eines Fadens oder seitlich an demselben und schwimmen im mikroskopischen Präparat umher als subglobose oder ellipsoidische, dickwandige, braune Zellen. Mitunter bilden sich auch zwei der-

Fig. 6. *Margarinomyces mutabilis.*

a. Konidienträger aus Agarkulturen. Vergr. 750 ×.
b. Konidienträger aus feuchte Kammerkultur. Vergr. 750 ×.
c. Konidien. Vergr. 750 ×.

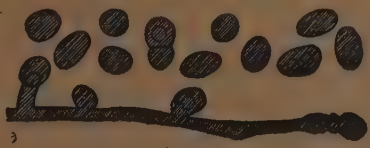

Fig. 7. *Margarinomyces mutabilis.*
Endständige Konidien. Vergr. 750 ×.

gleicher Zellen nacheinander, sie werden dann durch eine Querwand getrennt. Ein solches Gebilde stellt, frei geworden, eine zweizellige Konidie dar; diese kommen jedoch verhältnismässig selten vor. Eine Verwechslung mit *Phialophora mustea* Neergaard, die ebenfalls endständige Konidien in grosser Zahl erzeugt, ist ausgeschlossen, da letztgenannte auf allen Nährböden sofort eine schwarze Haut bildet, weil hier die dunkelfarbigen endständigen Konidien gleich vom Anfang an sich entwickeln.

Die Erzeugung von gewöhnlichen Konidien geschieht an mehr oder weniger flaschenförmigen, unverzweigten oder verzweigten Trägern, die entweder einzeln an den Hyphen entlang oder büschelartig beisammen stehen. Ein ganz anderes Bild ergibt die Kultur des Pilzes in einer feuchten Kammer. Dann werden, ähnlich wie bei *Pullularia pullulans* die Konidien in schneller Folge massenhaft an kleinen Vorsprüngen der fertilen Hyphen abgeschnürt, wonach sie sich zu Konidienköpfchen zusammenballen. Durch die Bildung der endständigen Konidien unterscheidet der Pilz sich von allen übrigen Arten dieser und der nahestehender Gattung *Phialophora* (5), weshalb die Beschreibung als neue Art unten folgt.

Margarinomyces mutabilis nov. spec.

Pilzdecke anfangs weiss, zottig durch aufstehende Hyphenbündel, später infolge der Bildung von endständigen Konidien schwärzlich.

Konidienträger mehr oder weniger flaschen- bis birnförmig, selten verzweigt, 10—20 μ lang, an der breitesten Stelle 2,7—4 μ dick, einzeln oder büschelweise an den fertilen Hyphen entlang stehend, die Konidien einzeln abschnürend.

Konidien massenhaft, einzellig, hyalin, ellipsoidisch, mit zwei Oeltröpfchen, (4,3—6,7) × (1,7—3) μ, meist (4,7—6) × (1,7—2,3) μ, in feuchter Umgebung auch unmittelbar an kleinen Ausstülpungen der Hyphen entstehend, zu kleinen Köpfchen verklebend.

Endständige Konidien in älteren Kulturen massenhaft, dickwandig, braun, glatt, fast undurchsichtig, oft mit 1—2 Vakuolen im Innern, meist einzellig, seltener zweizellig, akrogen oder pleurogen an den Hyphen entstehend, (6,3—10,7) × (5,3—6) μ gross.

Hab. Aus einer Flusswasserprobe (H. WINDISCH, München).

Reinkulturen:

Auf B i e r w ü r z e-A g a r in einer Petrischale nach 30 Tagen: Langsam sich ausbreitende Kolonien, 5 cm im Durchmesser, bestehend aus einer zähen Haut, überwachsen von zahlreichen kurzen, weissen Hyphenbündeln, welche die dunklere konidienbildende Schicht bedecken. Im Zentrum ein Polster aus grauen und weissen Hyphenbündeln, fast 1 cm hoch. Schwacher Geruch. Unterseite im Zentrum schwärzlich, sonst etwa 247.

Auf Röhrchen nach 10 Tagen:

Auf B i e r w ü r z e-A g a r: eine zottige, hellgelbbraune Kolonie, bestehend aus 1—3 mm hohen Hyphenbündeln, von denen die Konidienträger in grosser Zahl abgehen.

Auf K i r s c h-A g a r: wie vorige.

Auf M ö h r e: nur eine submerse, blassgelbe Haut ohne Konidienentwicklung.

Auf K a r t o f f e l s t ü c k: nur eine dünne, glatte, feuchte Haut ohne Konidienentwicklung.

Auf K a r t o f f e l-A g a r: eine zottige, gelbbraune Kolonie, bestehend aus 1—3 mm hohen Hyphenbündeln. Der Agar etwas gelb gefärbt.

Auf H a f e r f l o c k e n-A g a r: eine submerse, farblose Haut mit einzelnen, lockeren, farblosen Hyphenbündeln.

L a t e i n i s c h e B e s c h r e i b u n g.

Conidiophoris plus vel minus ampullaceis vel piriformibus, rariter ramatis, 10—20 μ longis, latissima parte 2,7—4 μ crassis, singulis vel fascinium in modum in hyphis fertilibus positis, conidia singula ferentibus. Conidiis numerosissimis, una cella consistentibus, hyalinis ellipsoideis, duabus oleaginis guttulis praeditis, (4,3—6,7) × (1,7—3) μ, plerumque (4,7—6) × (1,7—2,3) μ. Conidiis cacumine numerosissimis in vetustioribus culturis; crassis parietibus, brunneis, levibus, plerumque continuis, (6,3—10,7) × (5,3—6) μ.

Scopulariopsis capsici nov. spec.

Aus dem Laboratorium der „Specerijen-Malerij De Körver" in

Boxmeer (Niederl.) erhielten wir die Kultur einer *Scopulariopsis*, isoliert von Paprika (*Capsicum annuum* L.). Dieser Pilz besitzt ein langsames Wachstum auf allen Nährböden und bildet meist filzige, graubraune oder dunkelbraune, faltige Häute, von strähnigen, verfilzten, fertilen Hyphen überwachsen. Die Hyphen sind braun gefärbt, haben einen körnigen Inhalt und ein unregelmässiges Lumen. Sie treten gern zu dicken Hyphenbündeln zusammen, von welchen dann die Konidienträger dichtgedrängt allseitig abgehen. Die meisten Nährböden werden von dem Pilze dunkelbraun verfärbt. Die Beschreibung des Pilzes lautet folgendermaassen:

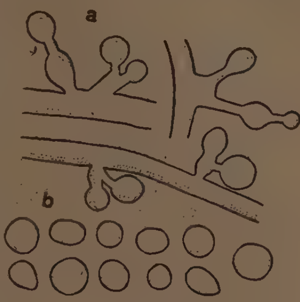

Fig. 8. *Scopulariopsis capsici*.
a. Konidienträger. Vergr. 750 ×.
b. Konidien. Vergr. 750 ×.

Scopulariopsis capsici nov. spec,

Konidienträger wenig verzweigt, meist aus einzelnen Sterigmen bestehend.

Sterigmen von unregelmässiger Gestalt: spielkegelförmig, gerade oder schwach gekrümmt, in der Mitte etwas aufgetrieben; oder breit ausladend, am Scheitel mehrere Konidien zugleich abschnürend; oder auch tonnenförmig, den Hyphen breit aufsitzend, 10—20 μ lang, an der breitesten Stelle 2—5 μ breit.

Konidien entweder eiförmig, d.h. an ihrem unteren Ende zugespitzt und am anderen Ende abgerundet oder subglobos, glatt, einzeln hyalin bis leicht gefärbt, in grossen Massen braun, oft mit kleinen Tröpfchen im Innern, in kurzen, leicht auseinander fallenden Ketten entstehend, 6—8 μ gross.

Perithezien wurden nicht beobachtet.

Hab. Isoliert von *Capsicum annuum* L. (Specerijen-Malerij De Körver, Boxmeer).

Reinkulturen:

Auf Bierwürze-Agar in einer Petrischale nach 8 Tagen: Langsam wachsend, Kolonie 1,5 × 1 cm im Durchmesser, wollig durch aufstehende, stark verfilzte Hyphensträhnen, welche die Konidien in grossen Massen erzeugen. Die Konidienfarbe ist graubraun. Um den Kolonien herum ein farbloser Saum, 0,5 mm breit. Kein Geruch. Unterseite etwa 268.

Auf Röhrchen nach 14 Tagen:

Auf Bierwürze-Agar: unten im Röhrchen eine kleine, flachwollige Kolonie mit zahlreichen Konidienträgern an den Hyphen, graubräunlich. Am Impfstück weisse, filzige Hyphenbündel. Agar braun gefärbt.

Auf Kirsch-Agar: am Impfstück entlang zahlreiche, hell-

graubräunliche, konidienbildende Hyphenbündel mit farblosen, kleinen Wassertröpfchen. Agar nicht gefärbt.

Auf M ö h r e: das Stück ganz bewachsen von einer glatten, feuchten, schwarzen Haut, von der sich zahlreiche braune, 1 mm hohe Hyphenbündel erheben.

Auf K a r t o f f e l s t ü c k: das Stück ganz bewachsen von einer glatten, trockenen, schwarzen Haut mit vereinzelten, aufstehenden, schwarzen Hyphenbündeln und grösseren, hellbraungrauen Konidienpolstern.

Auf R e i s: 2 cm tief gewachsen, die Körner überdeckt von grauen Konidienpolstern mit bräunlichem Stiche.

Auf H a f e r f l o c k e n-A g a r: eine submerse, schwarzgrüne Haut mit spärlicher Konidienbildung.

Lateinische Beschreibung.

Conidiophoris rariter ramatis, plerumque consistentibus ex singulis sterigmis. Sterigmis irregulariter formatis, rectis vel leviter curvatis, medio quodammodo intumescentibus 10—20 μ longis, latissima parte 3—5 μ crassis. Conidiis ovoideis vel subglobosis, hyalinis vel tenuiter coloratis, levibus; si numerosis brunneis 6—8 μ crassis.

Scopulariopsis croci nov. spec.

Das „Laboratorium voor Bloembollenonderzoek" in Lisse (Niederl.) schickte dem C. B. S. die Kultur einer *Scopulariopsis*, die von einer Crocus isoliert worden war. Auf allen Nährböden wächst der Pilz nur langsam an unter Bildung unebener Decken, welche eine puderige, braunschwarze Konidienschicht tragen, mit spärlichem Luftmyzel und einzelnen farblosen Wassertröpfchen. Die fertilen Hyphen schliessen sich gern zu Hyphenbündeln zusammen, von denen dann allseitig die Konidienträger abgehen. Letztere sind meist unverzweigt. Ihre Länge überschreitet meist nicht 15 μ, die Breite beträgt etwa 3—4 μ.

Fig. 9. *Scopulariopsis croci.*

a. Konidienträger. Vergr. 750 ×.
b. Konidien. Vergr. 750 ×.
c. Hyphenbündel mit Konidienträgern. Vergr. 245 ×.

Die Konidien werden in kurzen Ketten abgeschnürt, sie sind für eine *Scopulariopsis* ziemlich klein, nämlich etwa 4 μ im Durchmesser. Anfangs sind sie zitronenförmig, hyalin und glatt, im Alter runden sie sich ab und werden rauh oder stachelig und braun. Die Beschreibung des Pilzes lautet:

Scopulariopsis croci nov. spec.

Konidienträger meist unverzweigt, seltener verzweigt, einzeln an den fertilen Hyphen entlang stehend.

Sterigmen etwa von Spielkegelgestalt, 13—15 μ lang, an der breitesten Stelle 3—4 μ dick, meist einzeln stehend.

Konidien anfangs zitronenförmig, hyalin und glatt, später sich abrundend und rauh oder stachelig und braun werdend, 4—4,7 μ im Durchmesser.

Perithezien wurden nicht beobachtet.

Hab. Isoliert von einer Crocus-Varietät „Queen of the Blues", (Lab. voor Bloembollenonderzoek, Lisse, Niederl.).

Reinkulturen:

Auf Bierwürze-Agar in einer Petrischale nach 10 Tagen: Langsam wachsend, Kolonien 1 cm im Durchmesser, flach hügelig, von etwas wolligem Myzel überwachsen, im Zentrum braungrau, n.d. Rande hin heller. Rand filzig, weiss, 0,5 mm, in einen farblosen Saum übergehend. Kein Geruch. Unterseite mit 2 Zonen. Farbe etwa 297, im Zentrum oft etwas dunkler.

Auf Röhrchen nach 14 Tagen:

Auf Bierwürze-Agar: eine unregelmässig gewölbte und von kurzem Filz uberwachsene Decke, hellbraungrau. Rückseite dunkelbraun.

Auf Kirsch-Agar: wenig angewachsen, einige kleine, farblose, häutige Bildungen ohne Konidien.

Auf Möhre: das Stück ganz bewachsen von einer Haut mit rundlichen Auswüchsen, welche zum Teil farblos, zum Teil schwarz sind. An den braunschwarzen Stellen Konidienbildung.

Auf Kartoffelstück: das Stück ganz bewachsen von einer hellgraubraunen, glatten, feuchten Haut mit rundlichen Auswüchsen, worauf sich stellenweise braungraue Konidienpolster entwickelt haben. Das Stück etwas braungelb verfärbt.

Auf Reis: 1,5 cm tief gewachsen, an den Körnern braungraue Konidienpolster.

Auf Haferflocken-Agar: eine üppige Entwicklung von kurzen, filzigen Hyphenbündeln, hellgrau, mit reichlicher Konidienbildung und zahlreichen, farblosen Wassertröpfchen. Rückseite farblos.

Lateinische Beschreibung.

Conidiophoris non ramatis. Sterigmis conoideis 13—15 μ longis, latis ima parte 3—4 μ crassis. Conidiis primo citreniformibus, levibus, hyalinis, postea se conglobantibus, rudibus vel spineis atque brunneis esse incipientibus, 4—4,7 μ crassis.

Penicillium australicum (Olsen-Sopp) emend. van Beyma

Schon mehrere Jahre befindet sich in der Sammlung des C. B. S. ein *Penicillium*, eingesandt von Dr. H. Zach im Juli 1928, unter

der Bezeichnung *Penicillium australicum* (Kap Labor) Hann. Es fehlt also ein Autorname, denn Kap Labor ist das Laboratorium von JOHANN OLSEN-SOPP in Mjösen (Norwegen), während Hann., eine Abkürzung von Hannover, sich auf das Laboratorium von C. WEHMER bezieht. Wahrscheinlich hat ZACH das *Penicillium* von WEHMER bezogen und es seinerzeit dem C. B. S. zugeschickt. Auch in einer alten Pilzliste von 1924 von Prof. Dr. PRIBRAM's mikrobiologische Sammlung (vorm. KRAL's bakteriologisches Museum) in Wien ist unser *Penicillium* als *Penicillium australicum* (Kap) aufgeführt. Da es gerade in der letzten Zeit wiederholt von verschiedenen Substraten pflanzlicher Herkunft isoliert wurde, ist damit auch die Frage nach dem Autor wieder akut geworden. Ein Schreiben unserseits an das Kap-Laboratorium blieb unbeantwortet. Herr H. ROBAK in Bergen (Norwegen) hatte auf unsere Anfrage die Freundlichkeit mitzuteilen, dass OLSEN-SOPP schon vor einigen Jahren verstorben und das Kap-Laboratorium nach aller Wahrscheinlichkeit aufgehoben worden ist.

Es kann jedoch kaum ein Zweifel darüher bestehen, dass *Penicillium australicum* von OLSEN-SOPP isoliert wurde, wie die Bezeichnung Kap andeutet, wahrscheinlich ist er niemals dazu gekommen, eine Beschreibung des Pilzes zu veröffentlichen. THOM erwähnt in seiner Monographie (10) den Namen bloss im Inhaltsverzeichnis. Da *Penicillium australicum* jedoch eine gute Art darstellt, die auch hin und wieder mal im C. B. S. angefragt wird, ist es u.E. unbedingt notwendig, eine Beschreibung dieses Pilzes zu geben. Um unnötige Verwirrung zu vermeiden, halten wir es für angebracht, den Namen *australicum* beizubehalten und aus obengenannten Gründen OLSEN-SOPP als den mutmasslichen Entdecker aufzuführen. Die Herkunft des Pilzes wird wohl unaufgeklärt bleiben.

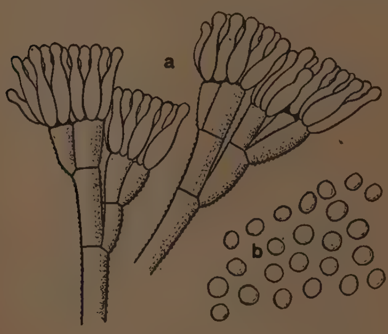

Fig. 10. *Penicillium australicum.*

a. Konidienträger. Vergr. 750 ×.
b. Konidien. Vergr. 750 ×.

Penicillium australicum gehört zu den schnellwüchsigen Penicillien. Die anfangs blaugrüne Decke färbt sich alsbald mehr reingrün und es treten Zonen auf. Typisch für den Pilz sind die ziemlich grossen, subglobosen Konidien, der Geruch nach Kartoffeln und der orangefarbene Saum auf Bierwürze-Agar um jede Kolonie herum. Die Beschreibung des Pilzes lautet folgendermaassen:

Penicillium australicum (Olsen-Sopp) emend. van Beyma.

Rasen stark puderig durch üppige Konidienbildung, 372—366—348. Deutlicher Schimmelgeruch, etwa wie Kartoffeln im Keller. Unterseite gelb. Um den Kolonien herum ein orangefarbener Saum, 1 mm breit, auf Bierwürze-Agar.

Konidienträger etwa 4 μ dick, rauh punktiert, verzweigt, mit 2—3 Sekundärästen, welche ihrerseits wieder je 2—3 Aestchen aufweisen, aus denen dann schliesslich die Sterigmen hervorgehen. Letzte Aeste 10—13 μ lang und 4 μ dick, je 3—5 Sterigmen tragend.

Sterigmen zahlreich, dicht gedrängt stehend, flaschenförmig, 13—15 μ lang und 3,3—4 μ dick.

Konidien subglobos bis kugelig, glatt, meist (4—4,7) × (3 — 4) μ gross.

Hab. Wiederholt isoliert von Pflanzenmaterial und aus der Erde (C. B. S., Baarn).

Reinkulturen:

Auf Bierwürze-Agar in einer Petrischale nach 5 Tagen: Kolonien flachwollig bis puderig, 372—366 oder etwas grüner, mit schmalem, weissem Rande. Deutlicher Schimmelgeruch. Um die Kolonien herum ein farbloser Saum, 2 mm breit. Kolonien im Zentrum etwas erhöht, körnig. Undeutliche Zonenbildung. Unterseite gelb, 221—216. Desgl. nach 10 Tagen: Decke puderig, fast ohne Rand, im Zentrum meist mit Myzelbüscheln, Farbe zwischen 347 und 342, nach dem Rande mehr 372. Grössere Kolonien mit radiären Falten. Rand körnig, weiss bis grünlich weiss, 1 mm. Saum 1—2 mm, orangefarben. Deutliche Zonen. Starker Geruch nach Kartoffeln. Unterseite im Zentrum 156—186, Rand 182.

Auf Röhrchen nach 7 Tagen:

Auf Bierwürze-Agar: Kolonien stark puderig bis puderig-wollig mit schmalem, weissem Rande. Rückseite 152—177—291.

Auf Kirsch-Agar: Kolonien puderig, blaugrün, etwas grüner wie 373. Unten im Röhrchen weisser Rand, 1,5 mm.

Auf Möhre: Ganz bewachsen, puderig mit vielen farblosen Wassertropfen, 368—348, oder etwas dunkler. Rand am Glase gelb, 221.

Auf Kartoffelstück: Fast ganz bewachsen, flachwollige bis filzige Haut, blaugrün, oben Farbe zwischen 368 und 373, oder stellenweise 367, ganz unten 366. Farblose Wassertröpfchen. Rand am Glase 221.

Auf Raulin: Decke flachwollig mit einigen Querfalten, oben weiss, unten 396.

Auf Reis: 1 cm tief gewachsen, oben blaugrün, 366—367—368. Der Reis stark gelb, 216—156. Am Glase 396.

Auf Haferflocken-Agar: mässiges Wachstum. Decke flach-puderig, 372—373.

Lateinische Beschreibung.

Conidiophoris plus minus 4 μ crassis, verruculosis, ramis praeditis, duos vel tres minores ramos gerentibus, duobus vel tribus ramiculis praeditis, quae 10—13 μ longa et 4 μ crassa sunt, quaeque singula 3—5 sterigmata gerunt. Sterigmatibus numerosis, densis, ampullaceis, 13—15 μ longis et 3,3—4 μ crassis. Conidiis subglobosis vel globosis, levibus plerumque (4—4,7) $\times$ (3 — 4) μ crassis, catenas firmas formantibus, odore mucido ethereo.

Zusammenfassung.

Es wurden folgende Pilze im Centraalbureau voor Schimmelcultures zu Baarn neu beschrieben: *Chaetomium minimum*, *Piptocephalis macrospora*, *Scopularia corsicana*, *Gliocladium cibotii*, *Margarinomyces mutabilis*, *Scopulariopsis capsici*, *Scopulariopsis croci*, *Penicillium australicum*.

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(From the Rijks Instituut voor de Volksgezondheid, Utrecht).

ON THE PREPARATION OF VACCINE AGAINST TYPHUS FEVER AND THE EXPERIENCES GATHERED THEREWITH [1])

by

A. PONDMAN

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The preparation of the vaccine against typhus fever does not differ in principle from the method along which the better known bacterial vaccines as those against typhoid fever, cholera, wooping cough are prepared. In fact in such cases it is aimed at to obtain a suspension of disease germs killed of by means of phenol or formaldehyde of such density, that a sufficient immunity may be provoked after a subcutaneous injection in man. Nevertheless for the preparation of vaccine quite another line must be taken up in case of typhus fever than for the above mentioned bacterial vaccines. As it is the causal agents of typhus fever, the Rickettsiae, do not belong to the bacteria, but may rather be reckoned among the viruses. They do not multiply (as far as we know up till now) on artificial media, but need for their growth the presence of living tissue. So new methods for the preparation of vaccine have been tried out with more or less success.

I will not go further into the various methods which have been worked out and which I have already discussed elsewhere (14), with the exception of a single one. Here a method of production of vaccine is concerned which up till now has proved very successful in practice, but which may not be applied in the Netherlands, the reason of which will be explained further down. A Polish investigator WEIGL (16) has contrived the nearest approach to the solution of the problem of the preparation of vaccine against typhus fever, which as a matter of fact is still in the experimental phase. He used as a culture medium for the multiplication of the Rickettsiae the lice (15). Nowadays it may be deemed well known that lice transmit the typhus fever, at least the disease occurring in East Europe. This insect has this property thanks to the fact that once infected with Rickettsiae these agents of disease can multiply at a quick rate in its body. They invade the endothelial cells of stomach and guts, where they multiply in such amounts that these cells will be filled

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[1]) Paper read in the meetings of November 21th, 1942 and November 15th, 1943 of the Nederlandsche Vereeniging voor Microbiologie.

with them next to bursting. Finally the endothelial cells will burst and their contents will be released in the lumen of the gut. At such a moment the faeces will consist chiefly out of Rickettsiae.

This process, which occurs under natural conditions after the louse has sucked blood of a patient of typhus fever some days before or during the febrile stage (6) where it gets infected with Rickettsiae, Weigl imitates artificially. In this end he infects the louse per anum with Rickettsiae containing material. The multiplication of Rickettsiae occurs then along the same line as described above, so that after a period of 5 to 8 days the lumen of the gut of these insects teems with Rickettsiae, out of which vaccine may be prepared.

Although this vaccine has been prepared on a large scale and its value has been established irrefutably, the above mode of preparation of vaccine cannot be applied in the Netherlands. The lice infected with Rickettsiae need as a matter of fact a period of 5—8 days to bring the disease germs to full development. During this period these strongly infective animals feed on human blood. Obviously persons, who consent to this treatment must be completely immune against typhus fever. Along with the sucking of blood by the lice the Rickettsiae are easily transmitted to man. In countries such as Poland, where this disease is of very frequent occurrence the finding of such immune persons does not offer difficulties. In a country free from typhus fever as which the Netherlands may fortunately still be considered, such persons are nor to be met with and thus this method for the preparation of vaccine cannot be applied.

As soon as it had been decided that the preparation of vaccine against typhus fever would be taken on hand, which under present conditions was assuredly indicated, some other method had to be followed. The chosing of a method out of the various existing ones was facilitated by the fact, that a method existed, which next to being relatively simple, had produced already good results. In this method, such as it has been described by Cox (3, 4), the developing fertile hen's egg is used as a nutrient medium for the growth of Rickettsiae. This method for the preparation of vaccine is frequently applied. In the ,,Staatliches Institut für Experimentelle Therapie" at Frankfurt this method has been applied successfully by Otto and Wohlrab (12). Gildemeister and Haagen (9) applied it as well, whilst Tchang and Mathews (18) judge this method very favourably in a communication from Peipin.

But before we could take the preparation of vaccine in hand, we needed to dispose of strains of typhus fever. These were not available in the Netherlands. Prof. Otto in Frankfurt had the kindness to hand over some to me. Along with the taking over of these strains, which took place in Frankfurt, I had the opportunity to become acquainted with the preparation of vaccine, an opportunity of which I made a grateful use.

By these means I obtained three strains of typhus fever, *viz.*, 2 originating from East European cases of typhus fever respec-

tively from Cracow and Warsaw, thus belonging to the type „Prowazek" and a strain originating from America which is to be reckoned to the type „Mooser".

For the preparation of vaccine the Rickettsiae are killed of with phenol or formaldehyde and only the Prowazeki strains can be used for this end. The less virulent and thus less dangerous Mooser strains cannot serve here as these Rickettsiae in living condition may induce sufficient immunity against the Prowazeki strains but not when dead [1]). And as the cases of typhus fever occurring in East Europe are caused by this race and as from those regions the danger for the Netherlands is most imminent, the Prowazeki strain had to be used for the preparation of vaccine.

These strains are kept active by animal passage, guinea pigs being the most suitable experimental animals (11). They are generally very susceptible for this infection. Usually it will not result in their death, but after the inoculation they will produce a typical fever curve. In the last two years we experienced that the susceptibility of the guinea pigs may strongly vary and depends on their batch of origin. A seasonal influence was hardly to be noted; we are of the opinion that it might rather be ascribed to a difference in susceptibility in the different breeds of guinea pigs. This was all the more probable as in the cultivation of the Mooser strains on mice we have been struck by a phenomenon of the same nature. The susceptibility of various breeds of mice appeared to vary from 0 % to 100 %. It is certainly noteworthy that information reached us from Tunesia about a 100 % susceptibility of the mice for Mooser strains, Frankfurt on the other hand mentions a susceptibility of 60 %, whilst here an average of merely 20 % was reached. Note has to be taken of the fact, that in Frankfurt and here a same Mooser strain has been worked with.

From a correspondence on this subject with Dr. BICKHARDT, Head of the Department for Typhus fever of the Staatliches Institut für Experimentelle Therapie at Frankfurt it appeared that the same Mooser strains, which WOHLRAB had taken over to Poland, remained there ineffective for mice. It appears to us, according to our limited experience, not improbable that hereditary factors are to be made responsible for this discrepancy in results. Under more normal conditions when we may dispose over more experimental animals we hope to gather more exact data.

For the preparation of the vaccine first of all the Rickettsiae have to be transmitted from the guinea pig into the fertile egg. As has been stated already guinea pigs produce a typical fever curve, which takes the following course: starting from the moment of inoculation the temperature initially will not offer anything notable.

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[1]) The preparation of the so called living vaccines in which the Rickettsiae have not been killed of has not been taken up by me, as it involves always dangers. I refer to a publication of BIRAUD (1) wherein for countries free from epidemics preference is given to „dead vaccines".

Now and then on the third or fourth day a slight raise in temperature may occur, which will fall to the norm again; suddenly on the sixth or seventh day it will rise steeply up till over 40° C. The temperature remains on this height during six to ten days and will then sink lytically. For the examination of the eggs (and moreover for the transmitting interperitoneally of the strain on the next guinea pig in view of the conservation of the strain) the infected guinea pig is killed on the fourth day of fever. It is supposed that at such a moment the Rickettsiae occur in greatest density in the brain substance. The brains are removed sterilly and brought in suspension in saline.

For the culture of Rickettsiae fertile eggs are used, which have been kept in the incubator at 39° C. at least five and at most eight days. During this period the eggs are viewed dayly, dead ones being discarded and the others turned over 180°.

Before the inoculation with Rickettsiae the eggs are washed with alcohol and then put up vertically with the air space in top. The area of the shell which covers the air space is painted with tincture of iodine. In its centre a small opening is made with a firm injection needle and the needle of the syringe is passed through. The syringe contains the above mentioned brain substance. The needle itself is pushed about 4 cm vertically into the egg and then slowly 0.5 ml of the content of the syringe is injected. The needle is drawn out of the egg and the opening of the shell is covered with paraffine.

As within 24 hours after the inoculation of the egg a great mortality occurred, it was tried to prevent this. Therefore first of all the dose of Rickettsiae of 0.5 ml as it was in use in Frankfurt, was brought down gradually. Obviously the percentage of the eggs which would take the infection might not decrease or at least as slightly as possible. Finally the amount of 0.2 ml was established, which allowed of a markedly lower „mortality within 24 hours", whilst the getting hold of the infection had not decreased worth mentioning.

In the first meeting in 1942 another factor was mentioned as possibly causing the early death of the embryos, *viz.*, the inoculation of the eggs in vertical and not in horizontal position. It was surmised that in vertical position the motile embryo would occur in the upper part of the egg and as a result be impaired by the injection needle. But it may plead against this that in the eggs which have lain some days in the incubator the lower surface of the air space is not even but bends downward convex. Thus the embryo will not occur medially under the air space but more obliquely; this can be observed during transillumination. In order to see more clearly into this two thousand eggs have been inoculated in vertical position against a same number in horizontal position, other conditions being equal. In this experiment no difference between both groups could be ascertained; in both the mortality was on a same level.

It ultimately appeared that in this lethal phenomenon another

factor played a major part, *viz.*, a toxine which will be discussed more in detail along with the egg to egg inoculation with Rickettsiae.

The fertile eggs after having been inoculated with Rickettsiae material are placed in an incubator at 37° C. Initially these eggs as well were viewed and turned over dayly, such as it has been described for the uninoculated eggs at 39° C. More recently this has been left of and the infected eggs are left completely undisturbed. The impression is gathered that in this way more eggs reach the final term of the period favourable for the cultivation of Rickettsiae.

When the eggs after this period are apparently still in living condition, they will be opened. This is practised by means of an electric burning apparatus, designed by SCHÜFFNER (17), a kind of thermocauter, by means of which the egg shell is burnt open along the limits of the air space. This method is assuredly to be preferred above the cutting open of the eggs. First of all sterility is guaranteed better, but moreover the experimentator is no longer in danger of infection. In fact along with the cutting minute particles of the infected shell may easily break of which may spread in all directions. GILDEMEISTER and HAAGEN (9), state in our opinion rightly, that the danger for the investigator actually occurs during the experiments with the eggs. Our experiences point in a same direction; only after the introduction of the eggs in the proceedings infection occurred.

The egg once opened its content is poured out in a sterile petri dish. Of the content of the egg only the yolk sac is used for the production of vaccine. It has been ascertained that other parts of the egg contain merely few Rickettsiae.

Two smears are made out of the yolk sac. They are stained and examined microscopically as to the amount of Rickettsiae and conclusions drawn as to the density of Rickettsiae in the yolk sac. Before staining the smears are dried, either during 24 hours at room temperature or about 30 minutes slightly heated. One smear is stained according to CASTANEDA, the other according to GIEMSA (as modified according to GRACIA MIGUEL).

The CASTANEDA staining consists in a staining with a buffered methylene blue solution (pH 7) and a counterstaining with safranine (2). After many failures, indicating that the pH 7 as mentioned for the stain is of chief significance, now hardly any more failures occur. Still the staining with the GIEMSA solution is always applied as well, as in the first place it is useful as a complimentary test and in the second place it is very favourable for the judging of the preparations.

The staining according to CASTANEDA, however, is much finer than the GIEMSA staining, as the former results in Rickettsiae stained a deep blue which contrast sharply with the pink underground, whilst in the latter case they contrast more slightly with their surroundings. Often in this case the Rickettsiae have to be recognized by their form, which requires a much larger practice. In

judging the values ascertained in the preparations, the classification as it has been put up by GILDEMEISTER and co-workers (9, 10) has been adopted, which comes down to the following:

$\pm$ = one single Rickettsia in the microscopic field
$+$ = 1 — 5 Rickettsiae per microscopic field (magnification 900 ×)
$++$ = 5 — 10 „ „ „ „ „ „
$+++$ = more than 10 Rickettsiae per microscopic field (magnification 900 ×)

Merely yolk sac smears which have been marked as $++$ or $+++$ may be used for preparation of vaccine. Needless to say that this method is hardly satisfactory, but as long as it has not been substituted by a better one, it will have to remain in use.

The yolk sacs are suspended in saline and these suspensions are ready for further egg inoculation, treated more in detail furtheron, whilst for the preparation of vaccine formaldehyde or phenol is added.

It is rare that at the first transmitting of Rickettsiae from the guinea pig into egg a sufficient density of Rickettsiae for the preparation of vaccine is attained at. Very often not a single one or at most a number of Rickettsiae marked merely as „+" will be revealed. When however further transmittings into egg are made, it may be noted that even in those cases where in the original material not a single Rickettsia had been detected, after repeated transmittings very rich cultures may develop. Along with this more profuse development of Rickettsiae the rate of growth will be seen to increase, so that it will no longer be needed to wait 10 days before opening the eggs; 5 days will suffice.

It might be expected that for further egg inoculations it would be needed to start from suspensions of yolk sac rich in Rickettsiae, *viz.*, from those marked „+ +" or „+ + +". This, however, is by no means the case. It has in fact been ascertained by GILDEMEISTER and HAAGEN (9) and as well by OTTO and BICKHARDT (13) that in material overgrown richly with Rickettsiae a toxine occurs which may kill of rapidly the newly inoculated eggs. The real nature of this toxine is up till now undefined. It appears noteworthy that this substance cannot be separated from the Rickettsiae by centrigugating, a fact which even renders the term „toxine" dubious.

It is obvious that this toxine was a source of difficulties in our initial experiments as we for transmitting actually made use of suspensions of yolk sacs heavily infected with Rickettsiae. Once acquainted with the cause of the difficulty it could be prevented in many cases. Its complete avoidance, however, is not possible, as it may occur sometimes that none but yields marked with $+++$ are available.

In order to keep down the loss as low as possible, two different roads were followed, *viz.*, either the liquid was diluted as far as possible, or the suspension was kept in the refrigerator at 4° C.

In the dilution method the number of Rickettsiae per unit of volume is diminished, which causes no difficulty as a suspension

marked „+" is already suitable for transmitting. But, and this is of more importance, the concentration of toxine is diluted, which means a lesser influence on the inoculated egg. In the refrigerator method as a matter of fact the same end is reached, the number of Rickettsiae per unit of volume decreasing along with a decrease in the concentration of toxine. In fact at low temperature, this very labile toxic substance is converted, at all events it disappears, so that without risking an early death of the embryo the eggs may be inoculated therewith. Either method has its advantages and disadvantages: it may happen that in the dilution method the influence of the toxine is not sufficiently weakened and in the refrigerator method that along with the toxine too many Rickettsiae are destroyed.

As has been stated the vaccine is prepared out of the „+ +" and „+ + +" yolk sac suspensions. In that end enough 5 % phenol is added to the suspensions to make up a 1 % phenol salt solution. Subsequently they are kept during eight days in a refrigerator at 4°C. in order to destroy any toxine eventually present.

Twelve yolk sac suspensions are mixed together. This is followed by a purification of the vaccine. These suspensions contain naturally many products valueless for the vaccine, which have to be removed as far as possible. This is done by centrifugating in the following way. Initially solid substances, so the Rickettsiae as well, are precipitated by means of centrifugating one hour on 8000 r.p.m. The supernatant valueless liquid is removed. The sedimentation is brought once again in suspension and centrifugated 10 minutes on 3000 r.p.m. By these means the coarser particles out of the sediment are precipitated. The supernatant liquid (now the sediment is discarded) in which the major part of the Rickettsiae occurs, is centrifugated over again one hour at 8000 r.p.m. The sediment thus obtained is subsequently brought in suspension in saline containing 0.5 % phenol in the ratio of 25 ml per yolk sac. The resulting product is the typhus fever vaccine, such as it is applied nowadays.

Obviously it will, however, have to pass several controls before it may be issued. Among these we mention: the control on sterility, the control on harmlessness, and finally the control on its immunizing value. As far as sterility is concerned, as a matter of fact all different stages of the vaccine are under control. First of all the sterility of the yolk sac suspension before phenol has been added, is ascertained in the usual way aerobically and anaerobically. Merely those yolk sac suspensions will be further worked with in which no contamination either by means of stained smears or by culturing could be ascertained. After phenol has been added the suspensions are kept during eight days in the refrigerator, followed by a control of sterility. No irregularities having been noted, after the twelve suspensions have been mixed together, again the sterility is ascertained. After centrifugation the sterility of the purified vaccine is tested once more. If sterile, the vaccine is distributed over ampullae and by means of a test at random the sterility is finally established.

The control of harmlessness consists in the intraperitoneal injection of the vaccine in 3 guinea pigs. These 3 animals may not during an interval of 3 weeks show any raise of temperature, which might remind of typhus fever. Initially we had gladly increased the number of animals, but under present conditions this could not be realised. The experience of recent years has taught, however, that 3 may suffice.

Simultaneously with the control of harmlessness the immunizing value is determined. In this end 5 guinea pigs are immunized with the vaccine. A month later these guinea pigs along with 5 normal animals (which as far as possible originate from the same batch as the immuzined) are injected intraperitoneally with Rickettsiae containing material. Moreover as far as possible unto these 10 guinea pigs 5 are added which had already been infected with typhus fever in an earlier experiment and had then shown a temperature curve specific for typhus fever. The results of the experiments on immunity, obtained at up till this moment, are presented in the following table.

	Experimental animals	Total number of guinea pigs	Typhus fever curve	Delayed typhus fever curve	No raise in temperature	Not got hold of infections in %
1.	Guinea pigs immunized with typhus fever	77	2	5	70	91
2.	Control guinea pigs	60	40 (4 dead)	13	7	11
3.	Reinfected guinea pigs	40	1*)	2**)	37	92
4.	Guinea pigs for transmitting of strain	739	517	91	70	9

*) Reinfection 5 weeks after the end of the first febrile period.
**) Reinfection 7 to 8 weeks after the end of the first febrile period.

Under 4 is inserted in this table a survey of the course of the infection in normal guinea pigs as it has been observed for the usual transmittings of the strains during the two later years.

It may be noted that in merely 9 % of normal guinea pigs the infection does not get hold. The percentage of 11 as inserted under 2 is thus on the high side; it may have been caused either by too small a number of animals in this group, or by a difference in susceptibility for the infection in the various batches of guinea pigs. Anyhow the 91 % out of the vaccinised guinea pigs in which the infection did not get hold shows clearly the immunizing action of

the vaccine. The more so as under 1 the results arrived at with the initially prepared vaccine are taken up as well. In the latter vaccines the demands on the control of value (number of Rickettsiae per microscopic field) had been less severe than in later experiments which will tell in the lesser immunizing effect. It thus may certainly be assumed that the 91 % as noted for the resistence against the infection is by no means flattered.

Moreover I wish to point to the fact, that in 5 of the immunized guinea pigs a delayed febrile curve occurred. In the non-treated animals this could merely be observed in 13 out of 53. Thus in immunized animals this delayed febrile curve is of more frequent occurrence.

Experience has taught that Rickettsiae which have been cultivated under unfavourable condition (too high or too low temperature) when injected into guinea pigs, may often induce a delayed febrile curve. The occurrence of such a temperature curve in vaccinised animals indicates in our opinion the fact that an inhibiting action is excercised on the Rickettsiae, which may lead to the conclusion of an existing immunity, although unsufficient, in these animals.

Was, however, a result of 100 % to be expected for the vaccination? To answer this question it has been inserted in the table under 3, what happened when guinea pigs which had already gone through a typhus fever were inoculated over new. In 3 out of 40 reinfected guinea pigs again a febrile curve arose; even the going through a typhus fever does not induce in guinea pigs 100 % immunity. Thus the answer to the above question has to be in the negative.

As far as the results of vaccination in man are concerned, we cannot give evidence based on experience of our own. We thus have to limit ourselves to literature.

In a publication of Ding (5) a summarizing discussion is to be found which presents a good survey of the results attained at up till now (see also Eyer (8)).

It appears that although vaccination against typhus fever does not decrease the number of cases, the disease takes in most of the cases a much less serious course than without vaccination. Finally special attention is drawn to the fact that no fatal cases occur among the vaccinised patients. The course of disease in two cases of typhus fever among our laboratory workers corresponds with this sentence.

Next to the preparation of egg vaccine the preparation of so called lung vaccine was taken on hand (7). The cultivation of Rickettsiae on mice lung did not offer technical difficulties. The growth was very profuse, often at least as profuse as in egg. After the lung had been ground down and phenol had been added a sterile vaccine could be obtained. It was, however, unsatisfactory that the sterility tests of the suspension of lung substance always resulted in the ascertainment of a contamination. It is true that it could be eliminated by means of phenol or eventually formaldehyde, but it seems

to us undesirable to make use of such a vaccine. As in the preparation of vaccine such as it has been described above, such contaminations are ruled out, the latter method is preferred above the preparation of the lung vaccine. Moreover it is much easier to dispose of a sufficient number of eggs than of mice. We mean to be all the more entitled to this conclusion in view of the following sentence in a publication of BIRAUD (1): „En effet, la méthode de culture sur embryons de poulets et celle sur poumons de rongeur sont également capables de fournir du vaccin en quantité et à l'heure actuelle, on ne peut pas prouver que le vaccin préparé par une de ces méthodes soit plus efficace que celui obtenue par l'autre".

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THE ANTIGENIC PROPERTIES OF BACTERIAL SPORES

by

J. H. BEKKER

(Received February 14, 1944).

A great number of researches have made it evident that a bacillus may be the bearer of various kinds of antigens, which, according to their properties, are designed as H-, O-, Vi-, L-antigens etc. Only a few authors have taken up the question whether the spores of the bacilli possess antigenic properties and ascertained whether an injection of spores into the blood of an animal produces antibodies specific for the spores and not for the bacillary forms.

The results of these experiments are contradictory. DEFALLE (2) working with *B.mycoides*, *B.mesentericus*, *B.subtilis*, *B.alvei* and two strains of low virulence of *B. anthracis*, MELLON and ANDERSON (5) working with *B. subtilis* and HOWIE and CRUICKSHANK (3) with *Cl. sporogenes*, *B. mesentericus* and two strains resembling *B. cereus*, are of the opinion that spores and bacilli each produce their specific antibodies, whereas STARIN and DACK (7) working with *Cl. botulinum*, *Cl. sporogenes* and *Cl. putrificum*, as well as KRAUSKOPF and McCOY (4) working with *B. niger* state that spores only produce antibodies to the bacillary forms.

I thought it interesting to investigate in a similar way a few different representatives of the genus *Bacillus*, *viz.*, an avirulent strain of *B. anthracis* (Bloed 1916), *B. ubiquitarius* and *B. mesentericus*.

TECHNIQUE.

In order to obtain asporogenic bacilli the strains were incubated on bouillon agar at 42° C. as previously described (1). Asporogenic strains were obtained of *B. anthracis* (Bloed 1916) and *B. ubiquitarius*, and the forming of spores could, if not entirely, still in a very considerable measure, be suppressed in *B. mesentericus*. H- and O-antisera were induced in the usual way in rabbits by means of *B. ubiquitarius* and *B. mesentericus*, whereas for *B. anthracis* (Bloed 1916) only an antiserum to the living bacilli was made.

Almost complete sporulation was obtained with *B. ubiquitarius* and *B. mesentericus* by incubating the cultures for 20 days at 37° C.

on asparaginate agar as described by HOWIE and CRUICKSHANK. As to *B. anthracis* (Bloed 1916) we detected in the films always some, though degenerated bacillary forms. Spore-antisera were prepared by 6 intravenous injections of 1 cc of a suspension of the growth of 5 asparaginate agars in 15 cc saline on successive days in a rabbit followed by 4 injections on successive days after an interval of a week; the animals were bled 4 days after the last injection. In a similar way spore-antisera were made by means of spores killed by autoclaving at 120° C. for half an hour.

Stable suspensions for the agglutination tests were obtained by a somewhat simplified technique of NOBLE (6) *viz.*, by shaking the bacterial suspensions in a mechanical shaker for 24 hours and allowing it to stand for 6 hours in which lapse of time the larger clumps will precipitate; the homogeneous supernatant liquid was used as suspension for the agglutination tests. For the spore suspensions these periods amount to 3 resp. 2 hours. The agglutination tests were incubated at 37° C. for 20 hours.

RESULTS.

The results of the experiments are given in the following tables:

Table 1

Strain: *Bacillus anthracis* (Bloed 1916)

Suspension \ Antiserum	living bacilli	living spores
living bacilli	1 : 640	1 : 40
living spores	nil	1 : 320

Table 2

Strain: *Bacillus ubiquitarius*

Suspension \ Antiserum	H-bacilli	O-bacilli	living spores	autoclaved spores
H-bacilli	1 : 640	nil	1 : 20	1 : 20
O-bacilli	1 : 20	1 : 320	nil	nil
living spores	1 : 20	nil	1 : 1280	1 : 640
autoclaved spores	nil	nil	1 : 320	1 : 320

Table 3

Strain: *Bacillus mesentericus*

Suspension \ Antiserum	H-bacilli	O-bacilli	living spores		autoclaved spores
			un-absorbed	absorbed with living bacilli	
H-bacilli	1 : 2560	nil	1 : 1280	nil	1 : 40
O-bacilli	nil	1 : 2560	1 : 40		1 : 80
living spores	1 : 80	1 : 80	1 : 1280	1 : 1280	1 : 640
autoclaved spores	nil	nil	1 : 1280		1 : 640

Table 4

Suspension \ Antiserum	living spores of *B.anthracis* (Bloed 1916)	living spores of *B.ubiquitarius*	living spores of *B.mesentericus*
living spores of *B.anthracis* (Bloed 1916)	1 : 320	1 : 20	1 : 20
living spores of *B.ubiquitarius*	1 : 20	1 : 640	1 : 40
living spores of *B.mesentericus*	nil	1 : 20	1 : 1280

Discussion.

From the tables 1, 2 and 3 it appears that the spores of the three strains tested possess antigenic properties. The spore antisera show besides the antibodies to the spores by means of which they have been prepared, no or only very few antibodies to the bacillary forms to which these spores give rise on germination. With *B. mesentericus* (Table 3) the living spore antiserum contained also many H-antibodies, possibly as a consequence of the small number of living bacilli occurring in the spore suspension or perhaps owing to the fact that some spores had germinated into bacilli in the body of the animal already. After absorption of this spore antiserum with living bacilli these antibodies had disappeared, whereas the spores were still agglutinated up to the titer.

Further it appeared that the bacillary antisera contained no or very few antibodies to the corresponding spores.

These facts may be considered as a proof for the existence of a specific spore-antigen that can clearly be distinguished from the antigens of the bacillary forms.

From the tables we can also see that autoclaving during half an

hour at 120° C. has little or no effect on the antigenic properties of the spores.

Finally I investigated whether the antigens of the tested spores differed mutually or whether there existed only one special spore-antigen. From table 4 it appears that the spores of the strain of *B. anthracis* (Bloed 1916), *B. ubiquitarius* and *B. mesentericus* produced antibodies distinct from each other, so that the spore-antigen of these strains are also distinct.

Summary.

1°. Bacterial spores are antigenic.
2°. Spore-antigen is distinct and separate from the antigens of the bacillary forms to which the spores give rise on germination.
3°. Antigens of the spores of various kinds of spore-bearing bacilli are also mutually distinct.

I am greatly indebted to Miss A. DE GROOT for her technical assistence.

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(Du laboratoire d'hygiène, Université de Groningue).

MÉTHODE SIMPLE POUR LA DESSICCATION DANS LE VIDE DE CULTURES BACTÉRIENNES

par

A. E. BEUTE

(Reçu le 17 Février 1944).

La dessiccation dans le vide et à température basse de produits biologiques, dans l' intention de les conserver, est en usage depuis longtemps et avec succès. Un résumé détaillé de la littérature a été donné e.a. par FLOSDORF et MUDD (3, 4).

Un appareil simple, construit principalement pour la dessiccation de cultures bactériennes, peut avoir une grande importance pour beaucoup de laboratoires, comme économie de temps et de milieux de culture. D'ailleurs les cultures séchées ne changent pas de nature; il n' y a ni dégénération, ni perte de virulence, conséquences bien connues et redoutées des repiquages répétés.

Avec le procédé en question ce sont surtout les détails pratiques qui sont intéressants. Voici une description brève de la méthode facile et de l'appareil simple, en usage depuis deux ans, pour la dessiccation de cultures bactériennes au laboratoire d'hygiène à Groningue.

L' appareil est basé sur le modèle de COOPER et GRABILL (2); cependant la construction et la méthode d'emploi sont assez modifiées. Ainsi le temps nécessaire pour la dessiccation a été ramené d'environ 24 heures jusqu' à une heure, l'appareil est plus robuste et surtout, en remplaçant la dessiccation d'une quantité de liquide par le dessiccation de bandelettes de carton buvard, trempées de liquide, la maniabilité est augmentée et les risques de contamination sont diminuées.

A p p a r e i l.

L'appareil à sécher se compose de deux parties principales:

1. La pompe à vide, munie d' un flacon de desséchage et des manomètres.
2. L'appareil à sécher proprement dit.

1. La pompe (a), une pompe à huile Cenco Hyvac, avec un flacon de desséchage (c) pour protéger l'huile contre les dernières traces d'humidité, et le moteur (b) sont montés sur une planche, ainsi qu'un manomètre fermé à mercure (d), un petit manomètre Mc Leod (e) et la conduite, servant à faire le vide, en cuivre (f). Presque

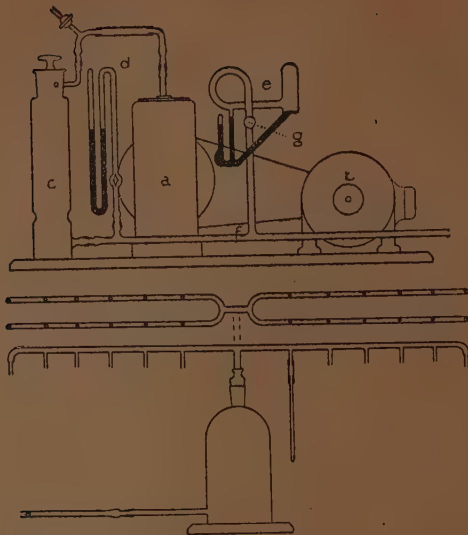

Fig. 1. Schéma au douzième environ. En haut la pompe à vide avec accessoires, en bas l'appareil à sécher propement dit.

toutes les jonctions sont des jonctions à l'émeri normalisées 11/10. Il est nécessaire d'employer pour le graissage des jonctions une graisse spéciale pour le vide; nous employons celle d'après Ramsay. Le bouchon à l'émeri du flacon de desséchage est perforé et sert de robinet entre la pompe et la conduite servant à faire le vide. Le tube de connexion entre la pompe et le flacon de desséchage porte un tube latéral avec robinet, pour donner accès à l'air. Le manomètre Mc Leod est un „drehbares Hochvacuummanometer nach Dr. Brunner" (1); fabrication de Bender et Hobein, München. Pour faire un mesurage, on tourne l'instrument maniable sur sa jonction à l'émeri (g). Les pressions peuvent être mesurées jusqu'à 0.001 mm.

2. L'appareil à sécher est en cuivre et se compose d'un vaisseau cylindrique et des bras porte-tubes. Le vaisseau a une hauteur de 18½ cm (jusqu'au col) et un diamètre de 12 cm. Dans la paroi, un peu au dessus du fond, un tube avec une jonction noyau à l' émeri 11/10, en cuivre jaune, est soudé, pour faire connexion avec la conduite à faire le vide. Le col du flacon est une jonction manchon à l'émeri 25/10 en cuivre jaune. Là s'emboitent les bras porte-tubes, avec des jonctions noyaux à l'émeri 25/10 en cuivre jaune. Les bras sont des tubes horizontaux, portant de petits tubes verticaux, munis de petites pièces de caoutchouc à paroi épaisse. Les

tubes de verre à évacuer peuvent être poussés dans ces dernières. Nous employons un porte-tubes avec un bras horizontal, muni de six tubes verticaux et un porte-tubes avec quatre bras horizontaux, munis au total de 24 tubes verticaux. Chaque tube horizontal est pourvu d'une petite tablette noire, pour écrire avec de la craie. En dehors de son emploi, le flacon est fermé d'un bouchon en cuivre jaune avec jonction à l'émeri 25/10. Le vaisseau en cuivre, comme le flacon de desséchage devant la pompe, sont remplis de drierite, du sulfate de calcium anhydre, fixant très vite l'eau, jusqu'au demi-hydrate. La tension de la vapeur de cet hydrate à 25° C. n'est que 0.004 mm. Drierite garde sa porosité en fixant l'eau. La régénération est très facile: en chauffant de 160 à 200° C.[1]).

Au lieu de drierite, on peut, probablement, se servir de silica-gel, mais nous ne l'avons pas expérimenté.

Méthode.

Chaque souche bactérienne est séchée au sextuple, pour avoir quelques réserves en faisant des cultures nouvelles.

L'emploi des milieux de culture solides est le plus simple. La culture d'un tube à milieu incliné, ou, en cas de cultures minces, de deux ou trois de ces tubes, est émulsionnée dans $\frac{1}{2}$ à $\frac{3}{4}$ cm³ de bouillon, de manière qu'une émulsion épaisse en résulte. Si la culture en milieu liquide est indispensable, il faut centrifuger les microbes et les émulsionner de nouveau dans $\frac{1}{2}$ cm³ du liquide surnageant. Ceci est facilité si le tube de centrifugation est pourvu par avance de trois perles de verre. Dans l'émulsion épaisse, dont la pureté est controlée au microscope et qui est transportée dans un petit tube stérile, sont trempées six petites bandes de carton buvard, d'une largeur de 25 × 3 mm et d'une épaisseur de 0.9 mm. Ce carton buvard doit être pur et neutre; nous employons la marque „Vampier''. Chacune des bandelettes, bien trempée de l'émulsion bactérienne, est transportée, avec une pincette dont les mords sont flambés, dans un tube de verre stérile, long de 16 cm, avec fond rond, tiré des tubes pour pipettes Pasteur (diamètre externe 0.6 à 0.8 cm). A l'aide d'une anse longue on pousse la bandelette jusqu'au fond. Le coton des tubes est coupé, de sorte qu' il en reste un demi cm; cette partie est rentrée un peu dans le tube.

On fait glisser les tubes, munis de bandelettes, à l'aide d'un peu de glycérine dans les pièces de caoutchouc de l'appareil à sécher.

Avec le porte-tubes aux quatre bras quatre souches peuvent être séchées à la fois. Un bloc de bois, pourvu de 4 rangées à 6 cavités, permet de manier facilement les 24 tubes, sans risquer de confusion. Sur ce bloc il y a une marge noircie, pour écrire avec de la craie.

Ensuite on fait le vide, la pression doit baisser au dessous de 0.2 à 0.3 mm de mercure; une bonne pompe atteint aisément quelques centièmes de mm. Une heure de desséchage suffit large-

[1]) Le produit est mis en circulation par W. A. Hammond, Yellow Springs, Ohio (U.S.A.). Représentant aux Pays Bas: Handelsonderneming v/h J. & W. Wegman, Staalkade 1, Amsterdam.

ment. Pendant ce temps on fait fonctionner la pompe. Au commencement on peut bien éprouver, à l'extérieur des tubes, le refroidissement des bandelettes et assez souvent le voir à une buée.

Après une heure on sépare par la fonte, en maintenant le vide, les 6 à 8 cm du fond des tubes, dans lesquels se trouvent les bandelettes. Pour cela est employé un petit brûleur avec deux flammes fines, croisantes, fait d'après les directions de FLOSDORF et MUDD(3). Pour le verre employé, de fonte facile, l'usage de l'air comprimé est superflu, même indésirable. La séparation exige un peu d'adresse. En commençant on fera bien de rétrécir un peu, à la flamme, les tubes à l'endroit de la fonte, avant de les fixer à l'appareil à sécher; après quelque exercise ce n'est plus nécessaire. Le verre ne doit pas coller des deux côtés, dans ce cas les tubes crèvent toujours après quelque temps; il faut chauffer uniformément de tous côtés, en tirant au fond du tube. Le bout est arrondi spécialement à la flamme.

Dans les tubes ayant une fuite, les microbes étaient toujours morts. On peut contrôler le vacuum des tubes avec un inducteur approprié; dans le vide il se produit une lueur violette.

Sur les tubes sont écrites, d'un pinceau fin et d'une laque à prise rapide, la signature de la souche et la date du séchage; les six tubes d'une souche sont réservés dans une boîte en fer blanc, comme on s'en sert pour expédier des matières infectées. Un petit billet ci inclus peut contenir des renseignements plus détaillés. Ainsi une souche, séchée au sextuple, ne demande pas plus de place qu'un tube de culture ordinaire.

Le vaisseau de cuivre contient 2 kg de drierite, pouvant fixer 132 g d'eau (6.6 %). Il vaut mieux n'utiliser que la moitié de cette capacité. Six bandelettes de carton buvard absorbent environ 0.4 g d'eau, de sorte qu'on peut sécher 165 souches bactériennes avant que la régénération du drierite devienne nécessaire. Cela se fait en chauffant le vaisseau ouvert pendant 12 heures de 160 à 180° C.

Une personne peut facilement sécher 12 souches par jour, chacune comprenant six tubes.

Nous avons réservé les souches séchées pour la plupart à la glacière; une partie plus petite est restée à la température ambiante, mais dans l'obscurité. Jusqu'à présent nous n'avons pas observé de résultats différents.

Si l'on désire une culture nouvelle d'une souche séchée, on flambe le bout d'un tube et on le casse avec une pince dont les mords sont flambés; puis on coupe le tube un demi cm au dessus de la bandelette. Avec une pincette aux mords stériles, la bandelette est transportée dans un milieu liquide ou dans l'eau de condensation d'un milieu solide. Après l'incubation nécessaire la culture s'est développée de nouveau; en cas d'un milieu solide on peut étendre les colonies qui se trouvent autour de la bandelette sur toute la surface du culot.

Nous avons séché de la manière décrite plus de 170 souches de *Haemophilus meningitidis* et *Haemophilus influenzae*, ainsi que

plusieurs souches des genres *Neisseria*, *Diplococcus*, *Streptococcus*, *Lactobacillus*, *Vibrio*, *Salmonella*, *Eberthella*, *Shigella*, *Listerella*, *Bacillus*, *Corynebacterium* et *Fusobacterium*, aussi des levures. Nous n'avons eu qu'un échec, à savoir chez les Leptospires. Du reste une nouvelle culture a toujours réussi à partir d'un tube bien scellé. Le résumé suivant montre les résultats obtenus en ce qui concerne la durée de la vie des souches séchées, jusqu'a présent:

Espèce bactérienne	Séché pendant	Conservé à
Haemophilus meningitidis et *Haemophilus influenzae*	17 à 24 mois	glacière et temp. ordinaire
Neisseria gonorrhoeae	16 ,,	glacière
Neisseria intracellularis	12 et 17 ,,	,,
Diplococcus pneumoniae	17 ,,	,,
Corynebacterium diphtheriae	15 ,,	,,
Vibrio comma	12 ,,	temp. ordinaire
Salmonella schotmuelleri	16 ,,	glacière
Shigella alcalescens	12 ,,	temp. ordinaire
B. megatherium' mutilat de DEN DOOREN DE JONG	12 ,,	glacière

Sommaire.

Dessiccation dans le vide de cultures bactériennes, par procédé simple et rapide.

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ABSTRACTS

A. Quispel, The mutual relations between algae and fungi in lichens. Diss. Groningen, 1943. Recueil des Trav. bot. Néerl. **40**, 413, 1943.

The lichen-symbiosis was investigated by means of experiments with pure cultures of the components. As lichen-algae some *Cystococcus* species were isolated, the only lichen-fungus investigated was *Xanthoriomyces parietinae*. As an orientation, however, a great many experiments were performed with the fungi which are living in symbiosis with the aerial algae *Pleurococcus* and *Apatococcus*, as it appeared that these fungi are closely related to true lichen-fungi, whilst their growth-velocity is much better. In consequence they are an excellent object for the study of the lichen-symbiosis. As far as possible the results obtained with the investigation of these fungi were tested upon *Xanthoriomyces*.

It appeared that the fungi did not develop in synthetic culture solutions without the addition of certain nutrilites (aneurin, β-alanin and other bios substances). The lichen-algae can provide the fungi with these nutrilites. These algae themselves were stimulated by the addition of asparagin, nicotinic acid and certain bios substances, when developing in organic culture solutions. In inorganic solutions a good development could only be obtained after the addition of a small amount of ascorbic acid. It is very probable that the lichen-fungi are able to stimulate the photosynthesis of the algae by the production of ascorbic acid or a related substance.

The fungi did not produce lichenic acids in cultures. On the other hand the alga *Apatococcus minor* synthesizes a remarkable metabolic product, called apatococcin, which most probably is related with certain aliphatic lichenic acids.

An investigation of the water-household of some lichens showed that the protective influence of the fungus against a desiccation of the algae is merely very small and can only be perceived when the desiccation is not too intense.

The final conclusion is that the lichen-symbiosis may be regarded as a „mutualistic symbiosis'' in which the exchange of nutrilites plays an important role. A. Q.

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